ENDOCRINOLOGY

VOLUME 63 SEPTEMBER, 1958 NUMBER 3

Copyright © 1958 by the Endocrine Society

THE EFFECTS OF CASTRATION AND ANDROGEN REPLACEMENT ON THE NUCLEIC ACID COMPOSITION, METABOLISM, AND ENZYMATIC CAPACITIES OF THE RAT VENTRAL PROSTATE

W. W. S. BUTLER, III AND ARTHUR L. SCHADE

U. S. Department of Health, Education, and Welfare, Public Health Service, National Cancer Institute and National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

ABSTRACT

The pentose nucleic acid/desoxyribose nucleic acid ratio for normal rat ventral prostate tissue of approximately 2.4 is lowered to 0.9 or less following castration or stilbestrol administration, while testosterone replacement returns the ratio to the "normal" value. Q_{O_2} of prostates from 6–15 weeks castrates is about $\frac{1}{5}$ that of the "normal" value and the RQ falls from 0.9 to 0.4. Testosterone treatment re-establishes the "normal" Q_{O_2} and RQ values. Zymohexase concentration of castrate tissue is lowered to 5–10% that of "normal," while the α-glycerophosphate dehydrogenase concentration doubles. The concentrations of both enzymes in prostrates of 20-day-old immature rats are similar to those of normal adults rather than of castrates. By injecting increasing amounts of testosterone in 4-week castrate mature rats and measuring the zymohexase activity of the prostate tissue after 72 hours treatment, a definite threshold is demonstrated below which the activity is at the castrate level and above which there is a 20-fold increase in zymohexase activity. The acid phosphatase activity of the prostate tissue per gram wet weight falls in the range of 8–10 King-Armstrong units and is not affected by age of rat, castration or by testosterone or stilbestrol administration.

THE ventral prostate of the rat is a glandular organ whose structure and functional capacity depend upon androgenic steroids. Its response to withdrawal of these hormones is a profound decrease in size and weight, and a change in consistency from a full, soft gland to a shrunken, fibrous one. Reversal of these changes is effected by androgen replacement. The histologic patterns of the normal and castrate glands were described by Moore, Price and Gallagher (1) who used the ventral prostate as a sensitive bio-indicator of androgenic activity.

Although the rat ventral prostate is an easily available, structurally dis-

Received December 13, 1957.

tinct organ composed primarily of a tall columnar epithelium whose cells are responsive to androgenic stimulation and thus eminently suitable for both metabolic and enzymatic *in vitro* investigations, it has not been extensively studied. Bern (2) determined the respiration of this glandular tissue in calcium free-Ringer phosphate solution, with and without addition of glucose or fructose, and noted that testosterone, except in a high concentration of 50 μg./ml. was without effect on the Q_{O_2} values. Alkaline phosphatase secretion by the normal ventral prostate (3) is greatly reduced after castration (4) but sustained by androgen replacement. The cytochrome oxidase, succinic dehydrogenase (5) and transaminase activities (6) of the prostatic tissue decrease following castration.

The purpose of this study is to add to our understanding of the cellular metabolism of the rat ventral prostate by establishing further metabolic and enzymatic indices of normal, castrate, and hormone-substituted prostatic tissue.

PRELIMINARY EXPERIMENTS

As preliminaries to the metabolic and enzymatic investigations, we made histological sections and nucleic acid analyses of representative ventral glands from normal, castrate, and androgen-treated castrated rats. For these and the following studies 3 months old rats weighing approximately 300 grams were employed. The average wet weight of the "normal" gland was 285 mg. Five days after castration the average weight decreased to 80 mg.; after a month, the value had fallen to 15 mg. One week of testosterone injections in such a castrate rat brought the weight of the gland back to 173 mg.—approximately a ten-fold increase.

Histologic sections of the normal adult ventral prostate, formed of large cells with abundant cytoplasm, showed acini filled with a protein-containing secretion. In contrast, sections of prostatic tissues from castrates or stilbestrol-treated rats, formed of small cells with scant cytoplasm, did not have secretion in the acini. For this reason the concentration of pentose nucleic acid per dry weight of tissue, as well as the ratio of pentose nucleic acid (PNA primarily of the cytoplasm) to desoxypentose nucleic acid (DNA of the nucleus), should be useful (7) as indices of the effect of castration or administration of androgen antagonists on the gland's secretory activity. Table 1 summarizes typical weight and nucleic acid data obtained from normal and treated adult rats.

The apparent increase in DNA per unit weight of gland with castration or stilbestrol administration reflects the relative decrease of the cytoplasmic elements. The inverse response of the PNA concentration of the tissue supports the same conclusion. Thus, the PNA/DNA ratio for normal ventral prostatic tissue of approximately 2.4 is rapidly reduced upon castration or stilbestrol administration, while testosterone replacement in a castrate returns the ratio to the "normal" value. Histologically,

TABLE 1. WEIGHT AND NUCLEIC ACID ANALYSES OF VENTRAL PROSTATES OF NORMAL AND TREATED ADULT RATS

Osborn-Mendel strain rats 3 to 4 months old		Body weight (gm.)	Prostate wet weight (mg.)	Dry weight %	DNA (μg. per mg. dry weight)	PNA (μg. per mg. dry weight)	PNA* / DNA
No.	Type						
1	Normal	311 (300–325)	285 (235–386)	19.6	20.2	48.5	2.41
3	Castrate, 1 week	288 (265–305)	80 (64– 90)	18.4	36.9	28.4	0.77
4	Castrate, 3 months	304 (287–350)	14 (12– 16)	20.2	47.8	21.8	0.45
5	Castrate, testosterone-treated 1 week	291 (264–326)	173 (154–193)	16.6	15.5	42.6	2.75
6	Normal, stilbestrol-treated 1 week	253 (250–255)	143 (120–182)	16.6	36.9	32.6	0.88

* Nucleic acids were extracted from the cells according to Schneider's procedure (8). Pentose nucleic acid (PNA) was determined colorimetrically through use of von Euler's phloroglucinol reaction (9) and desoxypentose nucleic acid (DNA) by Dische's diphenylamine procedure (10). Where range of values is not given, the single value represents an analysis of pooled tissue.

there is no apparent difference between castration and stilbestrol effects on the ventral prostate of the rat. The castrate gland which has been stimulated with testosterone can not be distinguished from normal gland. The same situation obtains with the nucleic acid analyses of these tissues.

METABOLIC STUDIES

In order to determine the value of the several metabolic quotients (Q_{O_2}, $Q^{N_2}_{CO_2}$, $Q^{N_2}_{lactic_2}$, and RQ) of the rat ventral prostate of normal, castrated, and hormonally stimulated animals for comparison with the associated histological and growth changes, we employed the usual appropriate Warburg manometric procedures (11). The investigations extended not only to the *in vivo* effects of the administrated hormonal agents but also to their *in vitro* action on excised prostatic tissue. Since the respiratory and glycolytic metabolism of the prostate gland reflects the activity and concentration of its constitutive enzymes, we chose for particular study zymohexase (aldolase) and α-glycerophosphate dehydrogenase as indicators of the glandular response to castration and hormonal treatment. Acid phosphatase levels were also determined.

Methods: Following removal of the ventral prostate from the rat, killed by cervical fracture, the gland was put on a piece of filter paper moistened with saline and ice-chilled until sufficient glandular material had accumulated for slicing. Usually no longer than a half-hour intervened between the times of excising the first gland and slicing it. The glands were cut with a Stadie-Riggs slicer[1] to give tissue pieces of approximately 0.5 mm. thick. The slices were rinsed in physiological saline at 0°–5° C and removed to pieces of filter paper (Whatman #50) for gross drying. They were then rolled on addi-

[1] Arthur H. Thomas Co., Philadelphia, Pennsylvania.

tional filter paper and weighed on a torsion balance. Such rinsing of the tissue was desirable to remove adhering cut cellular substances from the slices and assure greater uniformity in respirable material. An example of the data obtained from Q_{O_2} determinations of slices of ventral prostates from different batches of normal rats is given in Table 2. In addition to establishing the fact that 0.2% glucose in Krebs-Ringer phosphate (K-R.p.) buffer solution increases the Q_{O_2} value by 10–25%, these data give evidence of reasonably satisfactory duplication of results with the prostatic tissue prepared in the manner described.

Results: A summary of Q_{O_2} and RQ values, determined for ventral prostates removed from normal and castrated rats, is given in Table 3. Data illustrating the *in vitro* effect of testosterone on the Q_{O_2} and RQ of slices of both types of prostatic tissue are presented. The *in vivo* effect of testosterone administration to six-week castrates on the Q_{O_2} and RQ of the ventral prostate is also shown. Evaluation of these results leads to the following significant conclusions. (a) The respiration, in buffer or serum, of prostatic tissues from rats castrated for 6–16 weeks was reduced to an average of 24% of the normal value. The more extended the period of castration, the greater was the drop in Q_{O_2} values. These results agree, at least qualitatively, with previous observations (12, 13) on the decrease of the Q_{O_2} of surviving rat prostatic tissue following castration. (b) The RQ's of 6 to 16 week castrate ventral prostatic tissues, determined in serum, were severely reduced from the normal value of 0.9 to an average of 0.4. (c) The *in vitro* effect of testosterone on the respiration of prostatic tissue from normal rats was that of inhibition to the extent of 20–30% when the suspending medium employed was phosphate buffer [cf. (2)] but, in serum, testosterone was without effect. On prostatic tissues from castrates, testosterone in sera occasioned, on the average, a 50% drop in the already low Q_{O_2} values. (d) Testosterone added *in vitro* to either normal or castrate tissue failed to shift appreciably its RQ. (e) The Q_{O_2} of the prostatic tissue from testosterone-treated six-week castrates returned to the normal values in accord with previous findings (12). In addition, the RQ rose from its low pre-treatment value to that of normal ventral prostate. (f) Sera from castrated animals had no inhibitory effect on the Q_{O_2} of slices of normal or castrate prostatic tissue.

Determinations of the anaerobic glycolytic activity of the normal ven-

TABLE 2. Q_{O_2} DETERMINATIONS OF NORMAL RAT PROSTATIC TISSUE IN KREBS-RINGER PHOSPHATE BUFFER WITH AND WITHOUT 0.2% GLUCOSE, pH 7.4

Expt. No.	No. of rats	Q_{O_2}	
		With no addition	With 0.2% glucose added
1	6	10.7	13.6
2	12	9.4	11.5
3	8	10.1	11.2

Q_{O_2} first-hour values based on the mm.^{3} O_2 consumed/mg. dry wt. of tissue/hr. calculated on the dry weight of water-rinsed tissue obtained from the experimental flasks at the end of a 2 hour run.

TABLE 3. Q_{O_2} AND RQ OF NORMAL AND CASTRATE RAT VENTRAL PROSTATE TISSUES

Medium and additions[a]	Q_{O_2}				RQ			
	Normal		Castrate		Normal		Castrate	
	No. of rats	Values	No. of rats	Values	No. of rats	Values	No. of rats	Values
K-R.p. (Endogenous)	8[b]	10.1	60	2.8 (2.4–3.1)	12	0.67 (0.61–0.71)		
K-R.p. (Endogenous)			5[c]	10.0			5[c]	0.67
K-R.p.+Testosterone	8[b]	8.1	30	2.0				
K-R.c. (Endogenous)	6	11.1			6	0.67		
K-R.p.+0.2% Glucose	8[b]	11.2	30	2.2				
K-R.p.+0.2% Glucose+Testosterone	8[b]	7.5	30	2.3				
Normal Serum	28	11.0 (9.0–12.3)	16	5.1	28	0.89 (0.77–0.98)	16	0.57
Normal Serum			25[d]	2.4			25[d]	0.33
Normal Serum+Testosterone	8	11.0	31[d]	1.5	8	0.89	31[d]	0.44 (0.43 0.44)
Castrate Serum	16	11.3 (9.7–12.0)	16	4.0	8	0.87 (0.81–0.97)	16	0.21
Castrate Serum			29[d]	2.2			29[d]	0.43
Castrate Serum+Testosterone			16 30[d]	1.5 1.0			16 30[d]	0.51 0.52

[a] The usual manometric procedures were employed (11) with buffer as suspending medium. When sera were the suspending media, the Summerson manometric method served (12). The Q_{O_2} first-hour values were calculated on the basis of final dry weight of tissue from the respiration vessels. 100% O_2 was used as gas phase with Krebs-Ringer phosphate (K-R.p.), pH 7.4 medium; 95% O_2+5% CO_2 was used with Krebs-Ringer carbonate (KR.c.) and serum as media. All sera were made 0.1% with respect to glucose. Crystalline testosterone was added to tissues in excess of solubility, approximately 1 mg./ml. Except where indicated, all castrates were 6 weeks castrated.

[b] Prostatic tissues of these rats were used in an experiment to give the comparable Q_{O_2} values presented.

[c] These 6 weeks castrated rats received 10 mg. testosterone subcutaneously every other day for 5 days and were killed on day after last dose.

[d] These rats were 4 months castrated.

tral prostate were made manometrically and chemically (11) using tissue from six rats. The $Q_{CO_2}^{N_2}$ varied from 1.02 to 1.26 and the $Q_{lactic}^{N_2}$ from 0.47 to 0.56. The very low values of these quotients, particularly that of $Q_{lactic}^{N_2}$, indicate minimal anaerobic glycolytic activity by the ventral prostate. Aerobic glycolytic activity was similarly insignificant showing values from $Q_{lactic}^{O_2}$ of the same low order as found for $Q_{lactic}^{N_2}$.

ENZYMATIC STUDIES

The marked decrease in respiratory activity of prostatic tissues consequent upon castration of the rat and its return to normal levels after administration of testosterone made inquiry into an associated enzymatic activity of the gland of particular interest. For this purpose the zymohexase activity of prostatic tissue extracts was chosen because of its critical role in the production from hexosediphosphate of the common respirable substrate, pyruvic acid (15). The α-glycerophosphate dehydrogenase activity of the same extracts was also determined since other studies (16) had revealed a certain correspondence between the cellular concentration of this enzyme with that of zymohexase.

Methods: Zymohexase and α-glycerophosphate dehydrogenase were assayed (16) in saline supernatants from centrifuged 10% homogenates (10% wet weight of tissue, 90% weight of physiological saline) prepared by 5 minutes high-speed grinding in glass homogenizers cooled in an ice-bath. The efficiency of the initial extraction of the tissue was demonstrated by the failure of a second extraction to show more than 5% of the activity of the first. Using crystalline zymohexase, we established that the constituents of the extracts did not interfere with the quantitative estimation of their enzymatic activity.

Results and Discussion: The effect of castration upon the zymohexase and α-glycerophosphate dehydrogenase activities of prostate tissue and the response of the gland of castrated animals to testosterone, to stilbestrol,

TABLE 4. ENZYME ACTIVITY OF PROSTATES OF NORMAL, CASTRATED, AND CASTRATE-TREATED RATS

Enzyme	Normal		Castrated				8-day-treated 4-week castrates[d]		
			Weeks after castration[c]						
	Adult[a]	Immature[b]	4	5	6	7	Testosterone	Stilbestrol	Testosterone plus stilbestrol
Zymohexase[e]	108 (88–124)	110	10	7	5	9	104	7	88
α-Glycerophosphate dehydrogenase[f]	35 (26– 47)	33	51	65	43	62	46	62	43

[a] 24 rats provided the ventral prostate tissue to establish these average values.
[b] Eighteen 20-day-old rats, average weight = 28 grams. Average weight of prostate gland = 12.5 mg., compared with an average of 285 mg. for normal rats with a body weight of 311 grams.
[c] A minimum number of 10 castrated rats was used to obtain any one value.
[d] Eighteen 4-week-castrated rats were injected with 8 mg. of each hormone indicated every other day for eight days, then sacrificed on the ninth day to provide gland extracts.
[e] *W* units of activity of 10% homogenate extracts at 25° C.
[f] Units of activity based on $\Delta d^{1cm.}_{340m\mu}$. for ten minutes effected by 1 ml. of 10% homogenate extracts at 25° C.

and to testosterone plus stilbestrol with respect to these two enzymes were extensively investigated. Typical values obtained for both enzymatic activities are given in Table 4. From these results it can be seen that castration affects greatly the concentration of zymohexase in the gland tissue by lowering it to 5–10% that of the normal. Such loss was coincident with loss in gland weight and atrophy of the epithelial cells as revealed by histological examination. In some instances the 5% level was attained in 2 weeks following castration, in others this low concentration was found only after 4 weeks. In contrast to the zymohexase loss, the concentration of α-glycerophosphate dehydrogenase not only did not fall but consistently increased following castration. Since the gland weight from castration was reduced to approximately 5% of its normal weight by 7 weeks after castration, the absolute amount of the dehydrogenase on the whole gland basis had of course been decreased but to a lesser extent than the weight loss. The low Q_{O_2}, the low concentration of zymohexase and the relatively un-

anced level of dehydrogenase of prostates of the castrated animals compared to that of normals reflects the metabolic differences of the tissues which predominate in the glands under the two physiological conditions. These enzymatic changes may be compared to the already observed decreases in malic dehydrogenase, succinic dehydrogenase, and cytochrome oxidase in rat prostates following castration (5, 17).

The concentrations of both zymohexase and α-glycerophosphate dehydrogenase in the prostate glands of 20-day old immature rats were similar to those of normal adults rather than of castrates. This fact is of interest because of the sexual impotency of the immature animals and the relatively small weight of the prostate versus their total body weight (1:2240) as compared to the value of a similar ratio shown by adult animals (1:1090).

As shown in Table 4, testosterone replacement effected a return of zymohexase concentration in the prostate to a normal level while stilbestrol administration, as anticipated, had no demonstrable effect on the already low enzyme value. When testosterone was injected into the castrates along with stilbestrol, the zymohexase concentration in the prostates again returned to a near normal level. This result indicates that the effect of the testosterone on the target organ is independent of stilbestrol.

To determine whether the increase in zymohexase concentration in the ventral prostate of a testosterone-treated castrate rat is directly proportional to the amount of steroid injected, we administered subcutaneously various amounts of crystalline testosterone in corn oil to 8 groups of 3 four-week-castrated mature rats and after 72 hours determined the weight of the prostate gland and its zymohexase activity per mg. wet weight of tissue. Table 5 summarizes the results obtained. The zymohexase activity increases abruptly and markedly (10–20 fold), when the amount of injected hormone is increased from 0.1 to 0.2 mg. per rat. This increased enzymatic activity is paralleled, albeit on a much smaller scale, by the 2-fold wet weight increase of the gland. The zymohexase concentration of the prostate tissue from the castrated rat treated with 1.0 mg. of testosterone approximates that of the normal, although the total weight of the gland is only about 15% of its "normal" value after the single injection of testosterone. The threshold response to testosterone concentration in terms of zymohexase activity of prostate tissue in treated castrate rats is clearly demonstrated. The use of this threshold effect as an end point in biologic assay has not been explored.

The acid phosphatase activity of adult normal human prostatic tissue falls in the range of 522 to 2284 King-Armstrong units (18) per gram of fresh tissue. At puberty, the enzymic activity is about 70 units while at 1½ years of age it is of the order of 1 unit (19). The level of activity of acid phosphatase in the prostates of adult man, monkey (20, 21), and dog (22) decreases following castration or stilbestrol administration and increases upon androgen replacement in castrates. To compare the acid phosphatase

activity of the ventral prostate of rats with the prostates of these mammals, we determined the enzyme activity levels of normal immature rats (20 days old), of normal adult rats, of adult rats castrated for periods of time from 1 to 6 weeks, and of 6-week castrated adult rats treated subcutaneously with five 10 mg. doses of crystalline testosterone in propylene glycol every other day for 10 days.

In determining the acid phosphatase activity in terms of King-Armstrong units, we employed both whole saline homogenates of the gland as well as the supernatants obtained from these homogenates after centrifugation at 5° C for five minutes at 20,000 xg. Incubation of both the homogenates and the supernatants with the test reagents was carried out in

TABLE 5. THE EFFECT OF VARIOUS DOSES OF TESTOSTERONE ON THE WEIGHT AND ZYMOHEXASE ACTIVITY OF THE VENTRAL PROSTATE OF 4-WEEK CASTRATE MATURE RATS

Condition of animal	Testosterone, mg.	Average wgt. prostate, mg.	Zymohexase (W units of pooled tissue)
Control	—	285 (235–386)	108
Castrate	—	10 (6– 14)	4
Castrate	oil only	8.2 (8–8.5)	2
Castrate	0.05	11 (7– 13)	8
Castrate	0.10	13 (9– 16)	2
Castrate	0.20	22 (15– 34)	60
Castrate	0.50	21 (20– 27)	66
Castrate	1.00	31 (28– 32)	109
Castrate	10.00	50 (34– 70)	110

Testosterone administered as a single injection in one ml. of corn oil. Weight and enzyme activity determined 72 hours following testosterone administration.

double side-armed Warburg respiration vessels with shaking at 120 r.p.m. at 37° C for 30 minutes. The reaction was initiated at zero-time by addition of the homogenate or supernatant from one side-arm and was halted by addition of the Folin-Ciocalteau reagent from the other side-arm.

The results obtained with the homogenates from the several types of prostates studied showed that the acid phosphatase activity of all fell in the range of 8–11 King-Armstrong units. The supernatant values ranged from 3.2 to 5.0 units. From these data it may be concluded that (a) the acid phosphatase activity of ventral rat prostatic tissue is 1/50 to 1/200 that of adult human prostatic tissue; (b) the activity levels are not markedly affected by age of the rat when compared to humans at puberty and adulthood, and (c) the activity levels are not responsive to castration or androgen replacement in a castrated rat.

Acknowledgments

The authors wish to thank Dr. H. B. Levy for carrying out the reported nucleic acid analyses. The technical assistance of Mr. Jiro Oyama, Miss Patricia Michael, Mr. Robert Reinhart, and Mr. John Miller is gratefully acknowledged.

REFERENCES

1. Moore, C. R., D. Price and T. F. Gallagher: *Am. J. Anat.* **45:** 71. 1930.
2. Bern, H. A.: *J. Endocrinology* **9:** 312. 1953.
3. Huggins, C. and W. O. Webster: *J. Urol.* **59:** 258. 1948.
4. Meyer, R. K. and W. H. McShan: *Recent Progress in Hormone Research* **5:** 465. 1950.
5. Davis, J. W., R. K. Meyer and W. H. McShan: *Endocrinology* **44:** 1. 1949.
6. Awapara, J.: *Endocrinology* **51:** 75. 1952.
7. Caspersson, T.: *Symposia Soc. Exp. Biol.* **1:** 127. 1947.
8. Schneider, W. C.: *J. Biol. Chem.* **161:** 293. 1945.
9. Euler, H. von and L. Hahn: *Svensk Kem. Tidskr.* **58:** 251. 1946.
10. Dische, Z.: *Mikrochemie* **8:** 4. 1930.
11. Umbreit, W. W., R. H. Burris and J. F. Stauffer: Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minneapolis, 1949.
12. Nyden, S. J. and H. G. Williams-Ashman: *Am. J. Physiol.* **172:** 588. 1953.
13. Rudolph, G. G. and W. R. Starnes: *Am. J. Physiol.* **179:** 415. 1954.
14. Summerson, W. H.: *J. Biol. Chem.* **131:** 579. 1939.
15. Burk, D.: *Cold Spring Harbor Symposia on Quant. Biol.* **7:** 420. 1939.
16. Schade, A. L.: *Biochim. Biophys. Acta* **12:** 163. 1953.
17. Rudolph, G. G. and G. R. Meneely: *Feder. Proc.* **10:** 241. 1951.
18. King, E. J. and A. R. Armstrong: *Canad. Med. Assoc. J.* **31:** 376. 1934.
19. Gutman, A. B. and E. B. Gutman: *Proc. Soc. Exp. Biol. and Med.* **39:** 529. 1938.
20. Gutman, A. B. and E. B. Gutman: *Proc. Soc. Exp. Biol. and Med.* **41:** 277. 1939.
21. Huggins, C.: *The Harvey Lectures* **42:** 148. 1946–47.
22. Huggins, C. and C. V. Hodges: *Cancer Research* **1:** 293. 1941.

AN ATTEMPT TO INDUCE PRECOCIOUS SEXUAL BEHAVIOR IN MALE GUINEA PIGS BY INJECTIONS OF TESTOSTERONE PROPIONATE[1]

ARNOLD A. GERALL

Department of Anatomy, University of Kansas, Lawrence, Kansas

ABSTRACT

Beginning at the first or second day of life, dosages of 500 μg. of testosterone propionate per 100 gm. body weight were administered daily to intact and castrated guinea pigs. There was no statistically significant evidence that the intromission and ejaculation responses developed precociously in these animals, when compared with intact guinea pigs receiving sesame oil. These results differ from those obtained with the rat in which precocious sexual development has been produced by testosterone propionate treatment. It was concluded from the accumulated evidence that the guinea pig and rat do not respond similarly to testosterone propionate during post-natal development and maturity.

IN 1940 Stone (1) reported that male rats 22 to 26 days of age injected with testosterone propionate manifested copulatory behavior 20 days earlier than control animals. In a subsequent study Beach (2) found that copulatory behavior was advanced approximately 30 days in male rats 15 to 16 days old treated with testosterone propionate. In addition, 2 of the 4 treated rats ejaculated at 29 days of age, whereas two control animals 30 days old when the study terminated had not ejaculated.

Contrary to expectations based on these studies of the rat, Riss, Valenstein, Sinks and Young (3) found no unequivocal evidence for precocious development of sexual responses in guinea pigs injected with testosterone propionate. Their experiment differed from those of Stone and Beach not only in choice of experimental animals but also in their use of intraperitoneal rather than subcutaneous or intramuscular injections. When testosterone propionate is injected intraperitoneally, there is greater likelihood that it may be picked up by the portal circulatory system and inactivated in the liver, and as a result their animals may have received the hormone in insufficient amounts to affect sexual development. In addition, Riss, Valenstein, Sinks and Young used castrated rather than intact animals which as they noted, may respond differently to the exogenous hormone. In the present study both subcutaneous and intraperitoneal injec-

Received December 23, 1957.

[1] This investigation was supported by research grant M-504 (C_5) from the National Institute of Mental Health of the National Institutes of Health, Public Health Service; grant administered by Dr. William C. Young.

tions were administered to intact as well as castrated animals in an attempt to determine the efficacy of testosterone propionate in inducing precocious sexual behavior in the male guinea pig.

METHOD

On the day of birth, 50 guinea pigs from a genetically heterogeneous stock were assigned randomly to each of eight experimental groups. Within 2 days after birth the animals were castrated or left intact, and injections of testosterone propionate or sesame oil administered either intraperitoneally or subcutaneously were begun. Every animal was weighed and injected daily with 500 μg. of testosterone propionate per 100 gm. body weight.[2] All animals were caged with their mothers and litter mates until 25 days of age when they were placed in cages containing males. Seven of the animals were dropped from the study because of illness. Another 5 were found to have testicular fragments and their data were considered separately from those obtained with completely castrated subjects. A summary of the eight experimental groups is presented in Table 1.

The first 10 minute mating test was given when the male was between 12 and 15 days old. Five minutes after the male was placed in a 24″×24″×12″ rectangular box with a one-way vision screen an estrous female of similar size was placed with him. Five to 8 days intervened between each of the succeeding 9 tests.

RESULTS

Table 1 shows the time from birth until the first ejaculation or intromission occurred in a mating test. As would be expected, none of the cas-

TABLE 1. AVERAGE NUMBER OF WEEKS FROM BIRTH BEFORE THE APPEARANCE OF EJACULATION AND INTROMISSION RESPONSES

Routes of injection	Testosterone propionate				Sesame oil			
	I.P.		S.C.		I.P.		S.C.	
Castrated	Ejac.	Intro.	Ejac.	Intro.	Ejac.	Intro.	Ejac.	Intro
	N=4*		N=6		N=4		N=2	
	9.2	7.0	7.5	7.5	—	—	—	—
Intact	N=8		N=6		N=4		N=4	
	8.6	7.1	6.8	5.8	7.7	6.3	7.7	6.3

* Number of animals in each group.

trated animals receiving oil exhibited intromissions or ejaculations. Intact animals whether injected with sesame oil intraperitoneally or subcutaneously manifested the ejaculatory response at an average age of 7.7 weeks. The comparable group in the experiment reported by Riss, Valenstein, Sinks and Young exhibited the ejaculatory response at 7.9 weeks. In the present study only animals receiving testosterone propionate subcutaneously ejaculated on the average before 7.7 weeks. This difference produced

[2] Testosterone propionate was supplied by Ciba Pharmaceutical Products, Inc.

by method of injection (Table 1) is small and does not reach the .05% confidence level which had been selected for rejection of the null hypothesis.

As described by Beach (2) the complete copulatory pattern of the rat is probably most comparable to the sequence of responses observed in the guinea pig preceding and including intromission. Data in Table 1, which presents the average number of weeks between birth and intromission, indicate a slight but non-significant advancement of the intromission response in hormone-treated guinea pigs.

To obtain a measure of general level of sexual drive, the behavior of the animals during mating tests was recorded and scored according to procedures discussed by Young and Grunt (4). Roughly speaking, the computed score is related to the rate at which various sexual responses are displayed and to the time of occurrence of the ejaculatory response during the 10 minute test. In Figure 1, the average scores are shown for the first and second 5 weeks of the experiment. The only experimental group showing higher sexual drive than intact oil-injected animals was that receiving testosterone propionate subcutaneously and this difference, obtained only during the first 5 weeks of life, could be a chance occurrence.

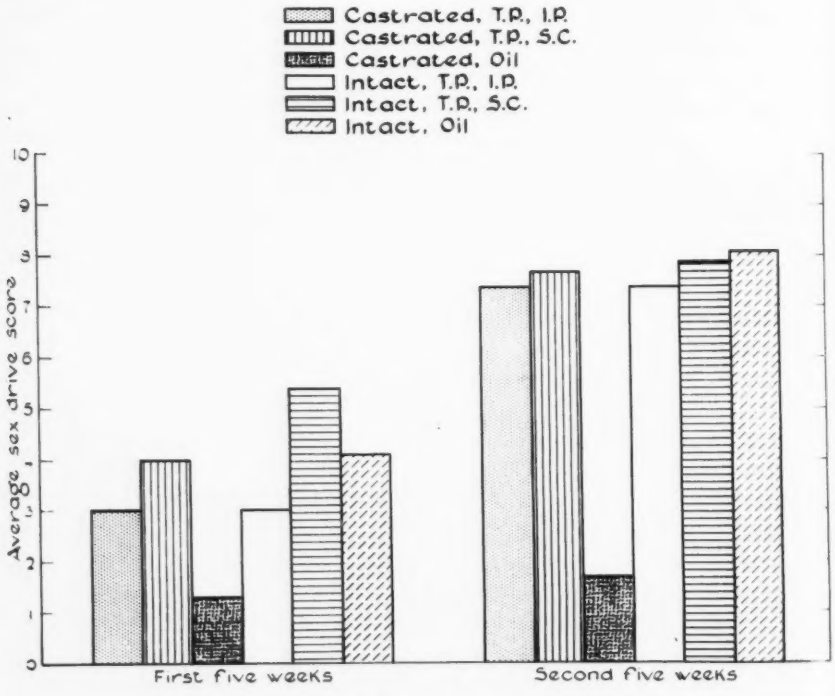


FIG. 1. Average sexual drive score during the first five and second five mating tests.

Two of the 5 guinea pigs containing testicular fragments received injections of oil. Their behavior was similar to that of the intact males. The 3 remaining animals were given testosterone propionate. The ages at which they manifested various sexual responses were similar to those of the completely castrated animals injected with testosterone propionate. The difference between this result and the report of precocity in two males containing testicular fragments and receiving 25 μg. of testosterone propionate and per 100 gm. body weight (3) cannot be explained.

DISCUSSION

In the present investigation an attempt was made to determine whether the routes of injection and the presence or absence of the gonads are factors that influence the effectiveness of testosterone propionate in accelerating the sexual development of the rat and guinea pig. Administration of testosterone propionate to intact and castrated guinea pigs via intraperitoneal and subcutaneous routes did not significantly advance the appearance of either the copulatory pattern or ejaculatory response. Nor were significant differences found in the level of sexual drive between experimental and normal animals. Therefore, it appears that during maturation the rat and guinea pig react differently to testosterone propionate treatment.

Not only the sexual maturation process, but also the level of sexual behavior and functioning of secondary reproductive organs in the adult guinea pig seem to be affected to a lesser extent by testosterone propionate treatment than in the rat. Stone (5) and Craig, Casida and Chapman (6) found that exogenous testosterone propionate can increase the sexual drive of some intact adult rats that had not mated prior to treatment. Attempts to elevate the level of sexual drive of intact guinea pigs by injections of testosterone propionate were unsuccessful (7). Also, when castrated rats were given supra-maintenance amounts of testosterone propionate sexual behavior was often superior to that exhibited before gonadectomy (8). In contrast, Grunt and Young (9) observed that the sexual drive of castrated guinea pigs was not raised above pre-operative levels by large dosages of exogenous hormone.

In both species injections of testosterone propionate produce greater than normal weights of the seminal vesicles, prostate and coagulating glands (10, 11, 12). However, an interesting difference between animals is reflected in the amount of secretory activity by the accessory reproductive organs following injections of testosterone propionate. Mann and Parsons (12) report that the level of fructose and citric acid in the prostate gland and seminal vesicles of rats injected with testosterone propionate was several times greater than that in normal rats. Mann (13) notes that injections of large doses of hormone in rats invariably were followed by "increased level of fructose formation well above that of non-treated controls." Recently, Ortiz, Price, Williams-Ashman and Banks (11) performed an

extensive study on the effect of testosterone propionate on the secretory activity of the accessory reproductive organs of the guinea pig. They concluded that the androgen did not produce precocious secretory activity in the organs of this animal.

Thus, testosterone propionate treatment accelerates the rate of maturation of sexual responses and produces greater than normal sexual drive and secretory activity of the accessory sexual organs in the rat, but it does not do so in the guinea pig. Judging from the results of the present experiment and others, it is doubtful that dosage level, routes of injection, age after birth, or castration effects are crucial factors.

One factor that might be important is the time measured from date of conception the injection of the hormone is started. The gestation period of the rat is 22 days whereas it is 68 days for the guinea pig. In studies with the rat, testosterone propionate was administered between 39 and 50 days after conception; when the guinea pig was used as a subject the hormone was not injected before 68 days after conception. Should the guinea pig receive the exogenous hormone at an earlier period in its development, the maturation of its sexual behavior might be influenced to an extent similar to that found in the rat.

REFERENCES

1. Stone, C. P.: *Endocrinology* **26:** 511. 1940.
2. Beach, F. A.: *J. Comp. Psychol.* **34:** 285. 1942.
3. Riss, W., E. S. Valenstein, J. Sinks and W. C. Young: *Endocrinology* **57:** 139. 1955.
4. Young, W. C. and J. A. Grunt: *J. Comp. and Physiol. Psychol.* **44:** 492. 1951.
5. Stone, C. P.: *J. Comp. and Physiol. Psychol.* **25:** 445. 1938.
6. Craig, J. V., L. E. Casida and A. B. Chapman: *Am. Nat.* **88:** 365. 1954.
7. Riss, W. and W. C. Young: *Endocrinology* **54:** 232. 1954.
8. Beach, F. A. and A. M. Holz-Tucker: *J. Comp. and Physiol. Psychol.* **42:** 433. 1949.
9. Grunt, J. A. and W. C. Young: *Endocrinology* **51:** 237. 1952.
10. Price, D.: *Physiol. Zool.* **17:** 377. 1944.
11. Ortiz, E., D. Price, H. G. Williams-Ashman and J. Banks: *Endocrinology* **59:** 479. 1956.
12. Mann, T. and U. Parsons: *Biochem. J.* **46:** 440. 1950.
13. Mann, T.: The Biochemistry of Semen.

BLOOD VOLUME CHANGES IN THE LACTATING RAT[1]

CHARLES F. BOND[2]

Department of Zoology, Cornell University, Ithaca, New York

ABSTRACT

Plasma volume (dye method), hematocrit value, and hemoglobin and plasma protein concentrations were measured in normal female rats at two stages of lactation and two weeks post-lactation, and from these data total blood and erythrocyte volumes and total hemoglobin were calculated. In the lactating rats, the total blood, erythrocyte and plasma volumes and total hemoglobin increased significantly above control values in non-pregnant rats, and returned to the pre-pregnancy levels in the post-lactation period. Plasma specific gravity decreased significantly during lactation, and there was a loss of plasma proteins in the second half of this period. Hematocrit values and hemoglobin concentrations did not rise above the control values. There was no correlation between maternal blood picture and litter size, but there was a high inverse correlation between litter size and the body weight of the offspring at the end of lactation. It is suggested that the increase in the volume of circulating erythrocytes constitutes a *lactation polycythemia*, the function of which may be to increase the oxygen-carrying capacity of the blood to meet the increased metabolic demands of lactation.

DURING pregnancy in the rat, the blood volume increases proportionally faster than the erythrocyte number which results in an apparent "anemia of pregnancy" (1), but during the lactation period, much less is known about the circulatory adjustments. Berlin *et al.* (2) reported total blood and erythrocyte volumes in three lactating rats without indicating the stage of lactation. The following report concerns changes in some blood constituents in rats during lactation and two weeks post-lactation. Plasma volume (dye method), hematocrit value, and hemoglobin and plasma protein concentrations were measured, and from these data were calculated total blood and erythrocyte volumes and total hemoglobin.

MATERIALS AND METHODS

Long-Evans nulliparous female rats weighing 180–210 grams initially were used. During pregnancy, lactation and post-lactation the rats were fed a standard calf meal ration *ad libitum*, and each rat was permitted to suckle all of its young.

Plasma volumes were measured by a T-1824 dye dilution technique; hematocrit

Received January 23, 1958.

[1] Aided by a grant from the Muscular Dystrophy Associations of America, Inc., and in part by the Sarah Manning Sage and Solon P. Sackett Research Funds of the Department of Zoology of Cornell University, administered by Dr. Samuel L. Leonard.

[2] Present address: Department of Zoology, University of Vermont, Burlington, Vermont.

values were obtained by using Van Allen tubes; plasma protein concentrations were calculated from plasma specific gravity measurements made with a LaMotte Falling Drop Densiometer, and hemoglobin concentrations were determined as acid hematin in a Fisher Electro-Hemometer. The procedures have been described in a previous paper (3). Total blood, erythrocyte and plasma volumes are presented as cc./100 gm. of body weight, and total hemoglobin as gm./100 gm. of body weight. Measurements were made on the 9th–10th and 20th–21st days of lactation and on the 13th–15th day post-lactation (dated from weaning on the 22nd–23rd day).

The data were analyzed by the small sample *t* test, and mean differences were considered significant if the value of P was 0.01 or less.

RESULTS

The data are presented in Table 1. The control values of the blood constituents in adult nulliparous rats from the same colony were used for comparison. Since it has previously been shown that the total blood volume on the 2nd day post-partum is the same as in non-pregnant females (1), it is evident that the total blood volume increased steadily during lactation and decreased following removal of the young. These changes were not merely concomitant with somatic growth since the body weight changed very little during the period from parturition through two weeks post-lactation. This constancy in body weight was contrary to the report of Cole and Hart (4), but their animals suckled only 4–6 young.

Hemoglobin concentration, total hemoglobin, and the volume of erythrocytes in circulation increased during lactation. Hemoglobin concentration and total hemoglobin on the 2nd day post-partum, on the basis of previous work (1), were approximately 12.2 gm./100 cc. of blood and 1.00 gm./100 gm. of body weight respectively. Therefore, in the first 10 days of lactation the total hemoglobin returned to the pre-pregnancy control value, and then increased significantly above this value in the second half of lactation. Two weeks after weaning the total hemoglobin value had returned to the control level. Hemoglobin concentration, on the other hand, increased during lactation but was not within the range of variation found in nulliparous females until late in lactation. The erythrocyte volume increased significantly during lactation, and had not yet returned to the pre-pregnancy level two weeks after removal of the young. Calculation of mean corpuscular hemoglobin concentrations (data not shown) revealed that the erythrocytes present in lactation were less rich in hemoglobin than the controls and that the hemoglobin concentration per erythrocyte gradually increased in the post-lactation period.

Plasma volume increased significantly during the first half of lactation and remained at this level in the second half. However, the plasma protein concentration decreased continuously throughout lactation. Since both hemodilution and an actual loss of plasma protein (possibly to the mammary gland) may contribute to the gradual decrease in plasma protein concentration, the changes in plasma volume suggest that hemodilution

TABLE 1. THE COMPOSITION OF THE BLOOD IN LACTATING FEMALE RATS

Group and number of rats	Day of lactation or post-lactation	Body weight (gm.)	Blood volume (cc./100 gm.)	Erythrocyte volume (cc./100 gm.)	Plasma volume (cc./100 gm.)	Hematocrit (%)	Hemoglobin concentration (gm./100 cc.)	Total hemoglobin (gm./100 gm.)	Plasma protein concentration (gm./100 cc.)
Non-bred controls (23)	—	197 ±4	8.1 ±0.1	3.4 ±0.1	4.7 ±0.1	42.4 ±0.5	14.3 ±0.1	1.16 ±0.01	6.52 ±0.10
Lactation[1] (10)	9–10	234 ±4	*9.1 ±0.1*	*3.8 ±0.1*	*5.4 ±0.1*	41.2 ±1.1	*12.6 ±0.4*	1.15 ±0.04	*5.93 ±0.10*
Lactation[1] (10)	20–21	228 ±4	*9.6 ±0.1*	*4.2 ±0.1*	*5.4 ±0.1*	43.6 ±0.6	13.7 ±0.3	*1.31 ±0.02*	*5.35 ±0.20*
Post-lactation[1] (10)	13–15	231 ±4	*8.7 ±0.1*	*3.7 ±0.1*	4.9 ±0.1	42.7 ±0.7	13.7 ±0.4	1.19 ±0.03	*5.83 ±0.13*

Mean values and standard errors are presented; those in italics are significantly different ($P < 0.01$) from appropriate controls (see text).
[1] The same animals in each instance; mean body weight on first day of pregnancy was 204 ±4 grams.

plays a great role in the first half of lactation and that plasma protein loss is more important during the second half. In the post-lactation period, the plasma volume decreased and the plasma protein concentration increased, but only the plasma volume was within the control range on the 13th–15th day.

The mean number of young suckled by the rats was 6.5, with 2 the minimum and 11 the maximum. On the 20th–21st day the mean body weight of the young was 32.5 gm. There was an inverse correlation (a coefficient of correlation of −0.86) between body weights of individual offspring and litter size, but there was no evidence of any correlation between litter size and the maternal blood picture.

DISCUSSION

In marked contrast with the extensive literature covering the condition classically known as the anemia of pregnancy is the relative paucity of literature pertaining to blood changes in the lactating rat. One reason for this apparent lack of information on the blood picture during lactation may be due to the results of earlier studies (5, 6, 7) which included only concentration measurements, that is, hemoglobin concentration, erthyrocyte count and hematocrit value. These findings, confirmed here, were interpreted as indicating a recovery during lactation from the anemia of pregnancy. However, in view of the great increase in total blood, erythrocyte and plasma volumes observed in the present study, a new interpretation of the blood picture in lactation appears justified.

It is suggested that the enlarged volume of circulating erythrocytes constitutes a *lactation polycythemia*, the function of which may be to increase the oxygen-carrying capacity of the blood to meet the increased metabolic demands of lactation. Recently, Fried *et al.* (8) stated that the rate of erythropoiesis is determined by the level of plasma erythropoietin and that erythropoietin production is regulated by the oxygen supply and demand relationship. It will be of interest to determine the amount of erythropoietin in the plasma of lactating rats.

The increase in plasma volume in lactating rats supports the hypothesis of Dieckmann and Wegner (9, 10) which states that an increase in plasma volume results in a lighter work load on the heart during human pregnancy and the early puerperium, two periods of metabolic stress. However, since the plasma volume did not increase after the middle of lactation while the erythrocyte volume increased throughout this period, the plasma volume increase in the rats was not merely a compensatory mechanism to prevent hemoconcentration. The results of some preliminary experiments by the author suggest that the cause of this plasma volume increase may be endocrine in origin, and additional experiments to test this hypothesis are in progress.

Although no attempt has been made here to determine how widely

applicable the lactation polycythemia hypothesis may be, it would appear valid in at least two species other than the rat. An extensive study of dairy cattle by Turner and Herman (11) revealed that the total amount of blood increased during lactation, which they attributed, in part, to enlargement of the udder. Reynolds (12) found an increase in plasma volume and a normal hematocrit value in lactating cows. Hansard *et al.* (13) discovered a 25% increase in total blood volume and a normal hematocrit value in lactating burros, but offered no explanation of their findings. In all of these studies the data indicate that polycythemia was present, and the normal hematocrit values show that erythrocyte and plasma volumes increased proportionally.

Acknowledgment

I thank Dr. Samuel L. Leonard of Cornell University for his gracious assistance in carrying out this investigation.

REFERENCES

1. Bond, C. F.: *Endocrinology* **43**: 180. 1958.
2. Berlin, N. I., R. L. Huff, D. C. Van Dyke and T. G. Hennessy: *Proc. Soc. Exper. Biol. & Med.* **71**: 176. 1949.
3. Bond, C. F. and S. L. Leonard: *Am. J. Physiol.* **191**: 296. 1957.
4. Cole, H. H. and G. H. Hart: *Am. J. Physiol.* **123**: 589. 1938.
5. Sure, B., M. C. Kik and D. J. Walker: *J. Nutrition* **1**: 299. 1929.
6. Mitchell, H. S. and L. Miller: *Am. J. Physiol.* **98**: 311. 1931.
7. Beard, H. H. and V. C. Myers: *Am. J. Physiol.* **106**: 449. 1933.
8. Fried, W., L. F. Plzak, L. O. Jacobson and E. Goldwasser: *Proc. Soc. Exper. Biol. & Med.* **94**: 237. 1957.
9. Dieckmann, W. M. and C. R. Wegner: *Arch. Int. Med.* **53**: 71. 1934.
10. Dieckmann, W. M. and C. R. Wegner: *Arch. Int. Med.* **53**: 188. 1934.
11. Turner, C. W. and H. A. Herman: *Mo. Agr. Exper. Sts. Res. Bull.* 159, 1931.
12. Reynolds, M.: *Am. J. Physiol.* **175**: 118. 1953.
13. Hansard, S. L., W. O. Butler, C. L. Comar and C. S. Hobbs: *J. Animal Science* **12**: 402. 1953.

EFFECTS OF CORTISONE ACETATE ON MILK PRODUCTION AND MAMMARY INVOLUTION IN PARTURIENT RATS[1]

ROBERT M. JOHNSON[2] AND JOSEPH MEITES

Department of Physiology and Pharmacology, Michigan State University, East Lansing, Michigan

ABSTRACT

Two similar experiments were performed on 68 parturient rats to determine the effects of cortisone acetate on lactation and mammary involution. The lactational response was measured by the effects on growth rate of the litters during the first 18 days postpartum. In the first experiment, 0.5 mg. of cortisone acetate daily elicited a significant increase in average litter weight gain over the control litters. A dose level of 1.0 mg. of cortisone acetate daily produced a slight but insignificant gain in average litter weight over control litters, despite a loss of over 15 per cent in body weight by the mother rats. In the second experiment, daily doses of 0.5 or 1.0 mg. of cortisone acetate, induced significant litter weight gains over control litters. When litters were removed on the 18th day postpartum, and cortisone acetate injections were continued for an additional 10 days, mammary involution was significantly retarded.

THE precise role of the adrenal cortex in the initiation and maintenance of lactation remains to be clarified. In hypophysectomized animals, adrenal cortical hormones are essential for the initiation and maintenance of lactation (1, 2). During pregnancy, adrenalectomy does not prevent the onset of milk secretion at parturition, but lactation is only of short duration (3, 4). Adrenal cortical activity is increased during lactation in the rat (5) and there is also evidence that the suckling stimulus elicits a release of adrenal cortical hormones, as indicated by a decrease in adrenal ascorbic acid and thymic involution (6, 7).

Most workers agree that injections of ACTH or glucocorticoids induce a prompt fall in established milk yields of cows (8–10), although one worker (11) had earlier reported an increase in milk production with ACTH. In intact or ovariectomized virgin rats, injections of ACTH, cortisone or hydrocortisone induce mammary growth, initiate milk secretion and in-

Received January 28, 1958.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2202.

[2] Submitted in partial fulfillment of requirements for the Ph.D. degree, Michigan State University, 1957. Present address: Department of Physiology, Colorado State University, Fort Collins, Colorado.

[3] We are indebted to Dr. L. Michaud of Merck, Sharp and Dohme, Rahway, N. J., for the cortisone acetate.

crease the prolactin content of the pituitary (12, 13). It was the purpose of the present experiment to determine the effects of cortisone acetate on milk production and mammary structure in the parturient rat.

MATERIALS AND METHODS

Two similar experiments were performed on 68 mature female albino rats. Once pregnancy was established, the dams were placed in individual cages. The feeding cups were designed to make the dam's ration inaccessible to the young. On the day of parturition the litters were reduced to 7 each in the first experiment and to 6 each in the second experiment. The mothers were treated in the first experiment as follows: 1, controls, physiological saline only; 2, 0.25 mg. cortisone acetate daily; 3, 0.5 mg. cortisone acetate daily; 4, 1.0 mg. cortisone acetate daily. The groups in the second experiment received the following treatments: 1 and 2, controls, physiological saline; 3, 0.5 mg. cortisone acetate daily; 4, 1.0 mg. cortisone acetate daily. All injections were administered subcutaneously in 0.1 cc. volumes for 18 days after parturition. The litters were weighed once daily and the dams once weekly until the end of the experiment.

In the second experiment, hormone injections were carried on for an additional 10 days after removal of the young on the 18th day, in order to determine their effects on mammary involution. Two groups of controls were used to follow the normal involutionary changes of the mammary glands from the 18th to 28th days. The right inguinal mammary gland was removed from each rat on the day of autopsy for histological examination.

RESULTS

In the first experiment the cortisone-treated dams (Groups 2, 3, 4) showed significant losses in body weight during the experimental period, which were related to the dose of cortisone acetate injected (Table 1). In contrast, the average body weight of the controls (Group 1) remained about the same during the experimental period. The adrenal weights of all

TABLE 1. EFFECTS OF CORTISONE ON BODY AND ADRENAL WEIGHTS OF DAMS AND ON BODY WEIGHTS OF LITTERS (Carworth rats)

Group and treatment	Av. dam wt. at parturition, gm.	Av. dam wt. at 18 days post-partum, gm.	Av. adrenal weight, mg.	Av. litter wt. at 18 days post-partum, gm.
1. Controls (9)* Saline Daily	244.5 ±7.0†	239.5 ±3.4	63.1 ±1.3	171.2 ±4.8
2. Cortisone (8) 0.25 mg. Daily	262.6 ±6.5	240.8 ±5.6	47.8 ±2.1	168.3 ±4.3
3. Cortisone (7) 0.5 mg. Daily	269.6 ±4.2	250.0 ±9.9	52.5 ±2.5	216.0 ±4.9
4. Cortisone (6) 1.0 mg. Daily	260.1 ±7.7	214.6 ±8.1	45.0 ±2.4	183.8 ±3.9

* Number of rats per group.
† Standard error of mean.

the rats receiving cortisone acetate were decreased as compared to the controls. The average litter weight gain was the same when 0.25 mg. of cortisone acetate was given daily (Group 2) as in the control litters (Group 1), but 0.5 mg. of cortisone acetate (Group 3) elicited significantly greater weight gains in the young. The average gain in litter weight of the dams given 1.0 mg. of cortisone daily (Group 4) was only slightly but not significantly greater than in the control litters, but this occurred despite a loss of more than 15% in body weight by the mother rats.

The results of the second experiment are shown in Table 2. Both doses of cortisone acetate (Groups 3 and 4) produced significant increases in average litter weight gain over the control litters (Groups 1 and 2). The inhibitory effects of cortisone acetate on the body weights of the mother rats of this strain were less marked than in the first experiment. Histological examination of the mammary glands from the control rats 10 days after the young were removed showed marked involutionary changes (Fig. 1). The alveoli were collapsed or in an advanced state of disintegration, and there was marked fibrosis of the lobules. Cortisone acetate, particularly when given at the 1.0 mg. level, significantly inhibited lobule-alveolar involution (Fig. 2). The alveolar epithelium was largely intact, and some secretory material was still present. These mammary glands were comparable to glands from control rats 5 days after removal of the litters.

DISCUSSION

These results indicate that cortisone acetate can increase established milk yields in rats, as measured by growth increment of their litters, and can also partially prevent mammary involution after the young are removed. This is in contrast to the inhibitory effects reported with ACTH or

TABLE 2. EFFECTS OF CORTISONE ON BODY WEIGHT OF DAMS AND LITTERS (M.S.U. rats)

Group and treatment	Av. dam wt. at parturition, gm.	Av. dam wt. at 18 days post-partum, gm.	Av. dam wt. at 28 days post-partum, gm.	Av. litter wt. at 18 days post-partum, gm.
1. Controls (9)* Saline Daily	262.2 ±5.0†	253.6 ±5.4	—	137.0 ±2.6
2. Controls (8) Saline Daily	237.8 ±7.7	233.7 ±7.5	239.7 ±7.1	142.5 ±2.8
3. Cortisone (8) 0.5 mg. Daily	252.0 ±7.9	242.0 ±5.6	238.1 ±6.0	172.3 ±4.6
4. Cortisone (8) 1.0 mg. Daily	247.0 ±7.4	226.8 ±7.5	222.1 ±6.4	163.7 ±4.1

* Number of rats per group.
† Standard error of mean.

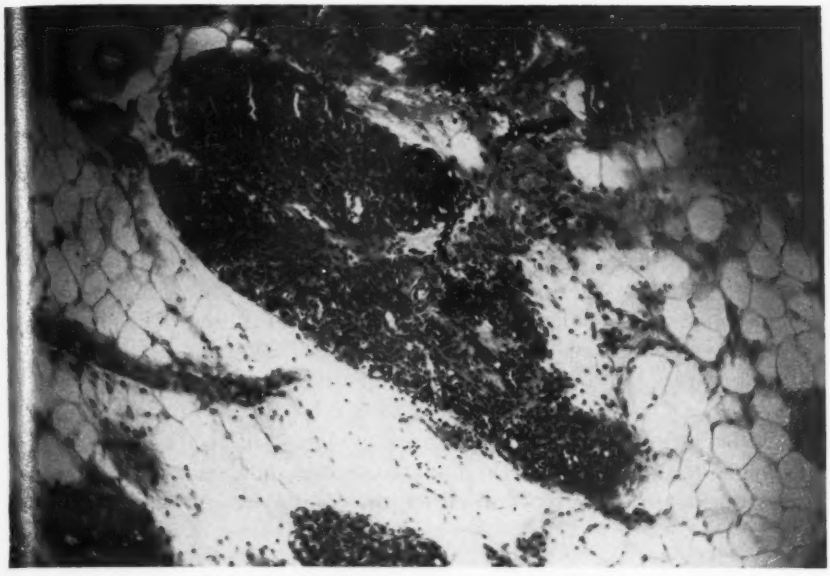


FIG. 1. Control mammary tissue from a rat 10 day after young were removed. (100×)

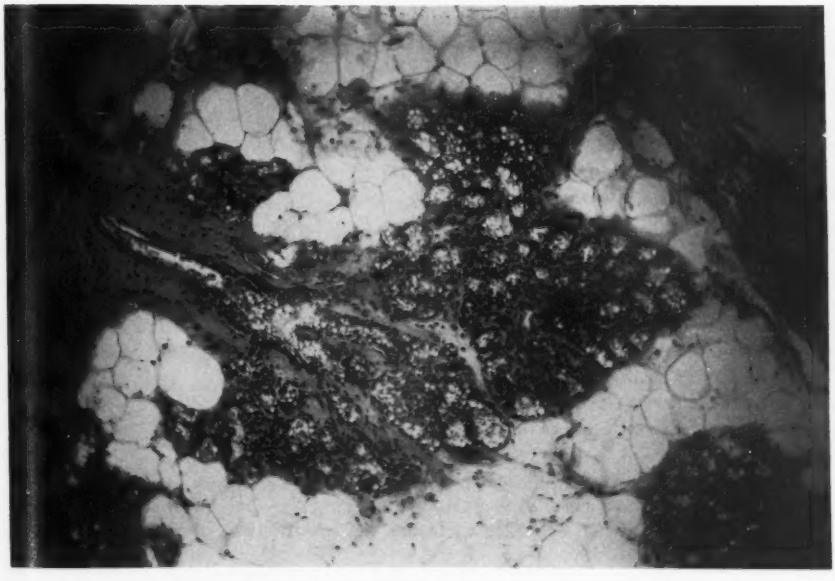


FIG. 2. Mammary tissue from a rat given 1.0 mg. cortisone acetate daily for 10 days after young were removed. (100×)

glucocorticoids on established lactation in cattle (8–10), but is in agreement with results reported in rats (12, 13). We have previously found that injections of 100 mg. of cortisone acetate daily into lactating goats failed to depress milk yields (unpublished results). Dosage factors as well as species differences may account for the contradictory actions of these substances on lactation in the cow and rat.

There is evidence that the suckling stimulus during lactation induces release of oxytocin (14–16), prolactin (17, 18) and ACTH (6, 7) from the pituitary. It has also been reported that injections of prolactin (19, 20) or oxytocin (20, 21) to lactating mother rats after removal of their litters inhibit involutionary changes in the mammary glands, and it has been suggested that oxytocin induces release of ACTH (22) and prolactin (21, 23) from the anterior pituitary. Oxytocin injections, however, have not been show to alter pituitary prolactin content in rats (18, 24). The present findings are believed to constitute additional evidence that the adrenal cortical hormones are important for the maintenance of milk secretion and mammary structure after parturition in the rat.

REFERENCES

1. Gomez, E. T. and C. W. Turner: *Proc. Soc. Exper. Biol. and Med.* **34:** 404. 1936.
2. Nelson, W. O. and R. Gaunt: *Proc. Soc. Exper. Biol. and Med.* **34:** 671. 1936.
3. Britton, S. W. and R. F. Kline: *Am. J. Physiol.* **115:** 627. 1936.
4. Meites, J., Trentin, J. J. and C. W. Turner: *Endocrinology* **31:** 607. 1942.
5. Poulton, B. R. and R. P. Reece: *Endocrinology* **61:** 217. 1957.
6. Tabachnick, I. A. and J. J. Trentin: *Fed. Proc.* **10:** 339. 1951.
7. Gregoire, C.: *J. Endocrinology* **5:** 68. 1946.
8. Cotes, P. M., Crichton, J. A., Folley, S. J. and F. G. Young: *Nature* **164:** 992. 1949.
9. Flux, D. S., Folley, S. J., and S. J. Rowland: *J. Endocrinology* **10:** 333. 1954.
10. Shaw, J. C., Chung, A. C. and I. Bunding: *Endocrinology* **3:** 327. 1955.
11. Roy, A.: Ph.D. Thesis. U. of London. 1947.
12. Selye, H.: *Acta Endocrinologica* **17:** 394. 1954.
13. Johnson, R. M. and J. Meites: *Proc. Soc. Exper. Biol. and Med.* **89:** 455. 1955.
14. Petersen, W. E. and T. M. Ludwick: *Fed. Proc.* **1:** 66. 1942.
15. Cross, B. A. and G. M. Harris: *Nature* **166:** 994. 1950.
16. Cross, B. A. and G. M. Harris: *J. Physiol.* **113:** 35. 1951.
17. Reece, R. P. and C. W. Turner: *Mo. Agr. Exper. Sta. Res. Bull.* 226. 1937.
18. Meites, J. and C. W. Turner: *Mo. Agr. Exper. Sta. Res. Bull.* 415 and 416. 1948.
19. Williams, W. L.: *Anat. Rec.* **93:** 171. 1945.
20. Johnson, R. M.: Ph.D. Thesis. Michigan State University. 1957.
21. Benson, G. K. and S. J. Folley: *Nature* **177:** 700. 1956.
22. Martini, L. and C. Morpurgo: *Nature* **175:** 1127. 1955.
23. Cowie, A. T. and S. J. Folley: In "The Neurohypophysis." H. Heller (Ed.). Academic Press Inc., New York, p. 183. 1957.
24. Johnson, R. M. and J. Meites: *J. Animal Science* **16:** 1045. 1957.

A NON-STEROIDAL ESTROGEN ANTAGONIST 1-(*p*-2-DIETHYLAMINOETHOXYPHENYL)-1-PHENYL-2-*p*-METHOXYPHENYL ETHANOL

LEONARD J. LERNER,[1] FRED J. HOLTHAUS, JR. AND CHARLES R. THOMPSON[2]

Scientific Division, The Wm. S. Merrell Co., Cincinnati 15, Ohio

ABSTRACT

MER-25, 1-(*p*-2-Diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenyl ethanol, blocks the response of estrogen target organs to the stimulating action of estrogens. The estrogen antagonism activity of MER-25 has been demonstrated in intact and castrate rats, mice and monkeys and in chicks and rabbits. The estrogen target organs employed for the measurement of the estrogen antagonism activity of MER-25 were the uterus, vagina and pituitaries in the mammals, and the oviduct in chicks. An additional endpoint employed was the chick plasma phospholipids. This drug antagonizes the activity of both endogenous and exogenous estrogens and steroidal and non-steroidal estrogens. The pituitaries of rats administered MER-25 were smaller than those of control animals and it prevented estrogen and castration induced pituitary hypertrophy. Weak gonadotrophin inhibition was evident in the treated male rat and in parabiotic female rats. MER-25 interfered with normal pregnancy in the rat. Male offspring of animals administered the drug during lactation were fertile but the female offspring were infertile. Studies described show that MER-25 does not have estrogenic, androgenic, anti-androgenic, progestational, anti-progestational or gonadotrophin-like activities.

INHIBITION or modification of target organ responses to estrogen by a number of natural and synthetic steroids has been reported. These steroids include estrogens (1, 2), progestational substances (3–8), androgens (3, 6, 9), adrenal cortical steroids and corticoids (4, 7, 9–18), and dehydrotachysterol (19). Response to estrogenic stimulation also can be impaired by creating a dietary deficiency of folic acid (20) or by employing a folic acid antagonist, aminopterin (21, 22).

The present report deals with a non-steroidal estrogen antagonist, 1-(*p*-2-Diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenyl ethanol, MER-25, which appears to block estrogenic response at the target organ site.

METHODS AND RESULTS

The mice employed in the following studies were obtained from the Maxfield Laboratory Animals Company, and the rats were obtained from the Rolfsmeyer Rat Company

Received January 28, 1958.

[1] Present Address: The Squibb Institute for Medical Research, New Brunswick, N. J.

[2] Present Address: Cutter Laboratories, Berkeley, California.

and Hamilton Laboratory Animals, Inc. These animals were provided with commercial rations and water ad libitum. Unless otherwise indicated, MER-25 was administered as a solution or suspension of the free base in olive oil.

Uterine Response in Immature Mice. Intact and castrate immature mice weighing 6–8 gm. were employed. In the studies in castrate animals, the mice were ovariectomized one day before starting the experiment. MER-25 was administered subcutaneously twice daily for three days to dose groups of five animals each, alone and in combination with an estrogen, testosterone propionate, progesterone or pregnant mare serum. The volume per injection for the olive oil solution or suspension of each of these compounds was 0.05 cc. The animals were sacrificed 17–20 hours following the last injection. The amount of fluid in the uteri was estimated, the uteri were removed, fluid expressed, and the uteri were weighed on a Roller-Smith balance.

Uterine responses to the subcutaneous administration of MER-25 are summarized in Table 1. MER-25 did not cause any uterine response in either castrate or intact mice in total doses of 5 mg. Total doses of 1 mg.

TABLE 1. UTERINE RESPONSE TO MER-25 IN IMMATURE MICE

Compound	Total dose	No. of mice	Uterus wt., mg.	Intrauterine fluid*
		Intact mice		
Control	—	37	7 ±0.3†	37N
Estradiol	0.3 μg.	37	39 ±1.8	36F, 1P
MER-25	5.0 mg.	38	8 ±0.3	38N
MER-25	1.0 mg.	34	11 ±0.4	1P, 33N
MER-25	0.2 mg.	25	10 ±0.7	25N
		Ovariectomized mice		
Control	—	10	7 ±0.6	10N
MER-25	5.0 mg.	10	8 ±0.3	10N
MER-25	1.0 mg.	10	10 ±0.6	10N
MER-25	0.2 mg.	10	7 ±0.4	10N

* F—Uterus distended with fluid.
P—Uterus partially filled with fluid.
N—No intrauterine fluid.
Number preceding letter indicates number of animals.
† Standard error of the mean.

produced a slight increase in uterine weight in both intact and castrate animals without causing any increase in the amount of uterine fluid. Doses of 0.2 mg. of MER-25 were similarly uterotrophic in intact mice but not in castrate mice. This increase was quite small when compared to that produced by 10 Mouse Units (0.3 μg.) of estradiol.[3] The histology of the uteri of animals administered a uterotrophic dose of MER-25 was similar to that of control animals and could only be differentiated from uteri of control animals by size and weight.

MER-25 blocked the uterotrophic effect of estradiol. It prevented both uterine weight increase and the fluid increase which occurs following the administration of estradiol (Fig. 1). The degree of antagonism depends on the dose of MER-25 and the dose of estradiol and the results indicate competitive antagonism. A dose of 5 mg. of MER-25 almost completely in-

[3] Estradiol represents estradiol monobenzoate.

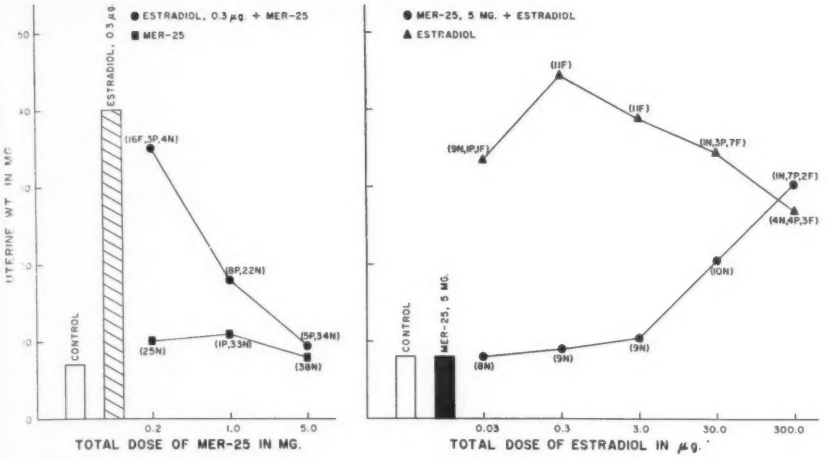


FIG. 1. Antagonism by MER-25 of the uterine effects of estradiol in immature mice. Numbers preceding letters at each dose point indicate number of animals per luminal fluid grade, and the letters indicate the grade (F = Full; P = Partial; N = No fluid).

hibited the uterine effects of doses of estradiol up to 100 M.U. (3 μg.) and partially prevented the uterine response to 1000 M.U. of estradiol. One mg. of MER-25 decreased the uterine weight increase produced by 10 M.U. of estradiol by more than 65%.

MER-25 prevented the uterotrophic activity of other steroidal and nonsteroidal estrogens (Fig. 2). Doses of 5 mg. of MER-25 prevented both

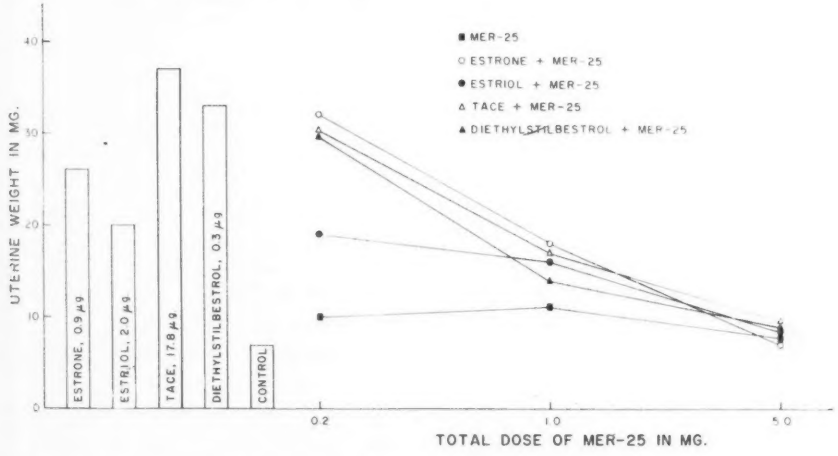


FIG. 2. Antagonism by MER-25 of uterotrophic effects of estrone, estriol, TACE (chlorotrianisene) and diethylstilbestrol in immature mice.

uterine weight increase and fluid increase which occurred following the administration of 10 M.U. of estrone, estriol, diethylstilbestrol and chlorotrianisene (TACE). The uterine responses to these estrogens were partially blocked by doses of 1 mg. of MER-25. Doses of 0.2 mg. had little or no effect upon the uterine response to the estrogens.

The decreased response of the uterus to estrogens when administered in combination with MER-25 is not caused by a delay in response to the estrogen. This was demonstrated in immature female mice. Following the administration of a subcutaneous dose of 1 mg. of MER-25 and 0.08 μg. of estradiol, no uterotrophic activity was observed even after five days (Fig. 3).

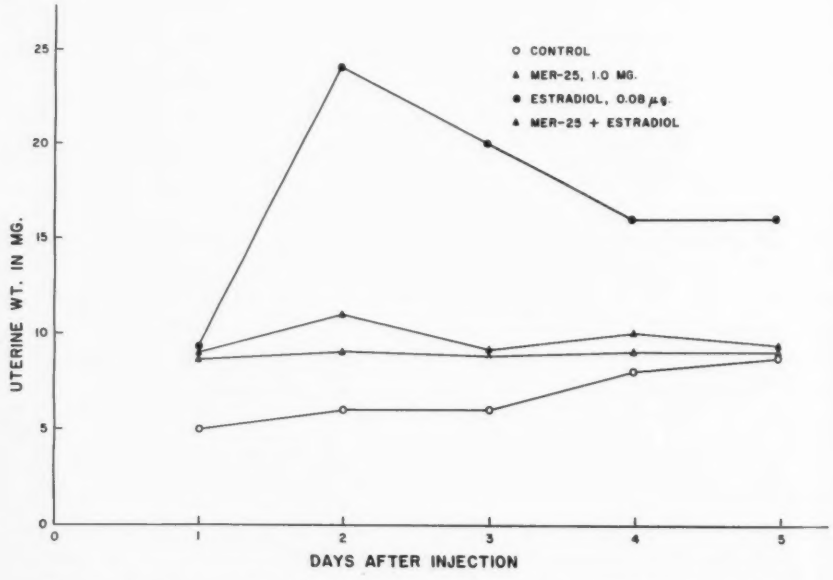


FIG. 3. Antagonism of a single subcutaneous injection of MER-25 to the uterotrophic effect of a single subcutaneous injection of estradiol in immature mice.

Results of experiments with testosterone propionate, progesterone, and P.M.S. (pregnant mare serum) are summarized in Table 2. The uterine response to testosterone propionate was not altered by MER-25 in doses up to 5 mg. The uterine response to progesterone was decreased by 5 mg. of MER-25.

P.M.S. induces uterine weight gain through its stimulation of ovarian secretions. MER-25 antagonized this effect at dose levels similar to those observed with exogenous estrogens. These results indicate, therefore, that MER-25 is equally effective against endogenous and exogenous estrogens.

TABLE 2. ANTAGONISM BY MER-25 OF THE UTEROTROPHIC ACTIVITY OF TESTOSTERONE PROPIONATE, PROGESTERONE, AND PMS IN IMMATURE FEMALE MICE

Treatment	Total dose	Uterus wt., mg.	Intrauterine fluid*
Control	—	7	5N
MER-25	5 mg.	8	6N
MER-25	1 mg.	12	6N
MER-25	0.2 mg.	9	5N
Testosterone propionate (T.P.)	0.5 mg.	19	5N
MER-25	5 mg.		
+T.P.	0.5 mg.	18	6N
MER-25	1 mg.		
+T.P.	0.5 mg.	24	5N
MER-25	0.2 mg.		
+T.P.	0.5 mg.	20	6N
Progesterone	2 mg.	17	6N
MER-25	5 mg.		
+progesterone	2 mg.	11	5N
MER-25	1 mg.		
+progesterone	2 mg.	17	5N
MER-25	0.2 mg.		
+progesterone	2 mg.	16	5N
PMS†	20 I.U.	31	4P, 2N
MER-25	5 mg.		
+PMS	20 I.U.	16	6N
MER-25	1 mg.		
+PMS	20 I.U.	26	1F, 5P
MER-25	0.2 mg.		
+PMS	20 I.U.	31	4F, 1P

* See footnote Table 1.
† Gonadogen—Upjohn Co. brand of pregnant mare serum.

Vaginal and Uterine Effects in Adult Castrate Mice. Adult mice were ovariectomized 2–4 weeks before starting the experiment. MER-25, estradiol or olive oil was administered subcutaneously at 8:30 A.M. and 4:00 P.M. of the initial day of the study. Vaginal smears were made at 48, 56, 72, 80 and 96 hours after the first injection. These smears were graded as positive only if cornified or nucleated epithelial cells were present in the absence of leucocytes. Immediately after making the final vaginal smears, the animals were sacrificed and the uteri removed, luminal fluid expressed and the uteri were weighed.

MER-25 in total doses up to 40 mg. did not produce a positive estrogen response in the vaginae of mice. It increased uterine weight at the total dose of 20 mg., but this uterotrophic effect was absent when the total dose was 40 mg. (Table 3).

Vaginal Effects in Adult Castrate Rats. Estrogenic and anti-estrogenic activities in castrate female rats were determined by the vaginal smear technique. Groups of 10 animals each were employed. MER-25 was administered subcutaneously and orally in two

TABLE 3. VAGINAL AND UTERINE RESPONSE IN OVARIECTOMIZED MICE

Compound	Total dose	No. of mice	Uterus wt., mg.	% mice with positive response
Control	—	10	13.7	0
Estradiol	0.15 μg.	10	39.6	100
MER-25	40 mg.	5	15.3	0
MER-25	20 mg.	5	24.8	0
MER-25	10 mg.	5	—	0

doses 8 hours apart, alone, and in combination with estradiol. Estradiol in an olive oil solution was administered in a single subcutaneous injection at the time of the first dose of MER-25. Vaginal smears were obtained 56, 64 and 72 hours after the administration of MER-25. Smears containing nucleated or cornified epithelial cells and only a few or no leucocytes were considered an estrogenic smear.

The vaginal smears of animals administered MER-25 in doses up to 50 mg. orally and 25 mg. subcutaneously were of the castrate type. Estradiol in doses of 0.6 μg. and 3.5 μg. (approximately 2 and 10 M.U.) caused vaginal cornification in 90–100% of the animals. The administration of MER-25 to rats treated with estradiol prevented the estradiol-induced estrogenic vaginal changes. Doses of 1 mg. subcutaneously and 2 mg. orally antagonized completely the vaginal responses to 0.6 μg. estradiol. Vaginal cornification was observed in 1/10 of the animals administered 0.6 μg. of estradiol in conjunction with 0.2 mg. of MER-25 subcutaneously or 1.0 mg. MER-25 orally. Smaller subcutaneous or oral doses of MER-25 had little or no effect against 0.6 μg. of estradiol. Larger doses of estradiol were effectively antagonized by larger doses of MER-25 (Table 4).

Effect upon Vaginal Cornification of Intact Rats. The effect of MER-25 on the vaginal estrous cycles of young mature rats was determined by daily vaginal smears. A group of 9 rats was administered MER-25 by stomach tube in doses of 1 mg./day for 14 days and in doses of 5 mg./day during the next 17 days. A group of 10 rats was administered olive oil by stomach tube. Five animals from each of the groups were sacrificed on the

TABLE 4. ESTROGENIC AND ANTI-ESTROGENIC VAGINAL ACTIVITY IN CASTRATE FEMALE RATS

Total dose of compound		% rats with positive estrogenic response
MER-25, mg.	Estradiol, μg.	
Subcutaneous MER-25 administration		
—	—	0
—	0.6	100
—	3.5	100
25	—	0
5	—	0
1	—	0
0.2	—	0
0.04	—	0
25	0.6	0
5	0.6	0
1	0.6	0
0.2	0.6	10
0.04	0.6	90
Oral MER-25 administration		
50	—	0
10	—	0
2	—	0
50	0.6	0
10	0.6	0
2	0.6	0
1	0.6	10
0.4	0.6	70
10	3.5	0
2	3.5	80

TABLE 5. VAGINAL ESTROUS CYCLES OF INTACT FEMALE RATS

	Rat No.	Length of consecutive cycles in days: Pre-drug period	MER-25 treatment period: 1 mg./day ×14	MER-25 treatment period: 5 mg./day ×17 days	Day of first post drug estrous smear	Post drug period
MER-25 treatment group	1	5-7-5	7-4-6	18 days without cycles		
	2	5	5-5-6	18 days without cycles		
	3	13-4	7-4	24 days without cycles	Animals sacrificed on first day post drug	
	4	6-4-5	6-5-4	17 days without cycles		
	5	5-6-4-5	6-5	22 days without cycles		
	6	9-6-4	6-5	16 days without cycles	6	4-6-4
	7	6-6-5	5-4-6	19 days without cycles	4	6-3-5
	8	6-4-5-5	11-3	17 days without cycles	2	4-4-5-5
	9	15-5	6-5-6	21 days without cycles	2	4-4-5-5
Control Group	10	6-5-4	5-5-6	4-4-5-6		
	11	5-7-5-4	6-4-4	6-5-5	Animals sacrificed on day corresponding to first day of post drug period	
	12	11	5-4-5-5	6-5-4		
	13	9-7	6-5-5	5-6-5		
	14	5-5-5	6-4-6	4-5-6		
Cycles corresponding to experimental periods of treatment group	15	6-5-4	5-5-6	5-3-7		4-6-5-5
	16	6-6	4-5-5-6	4-6		5-4-6-3
	17	6-4	5-6-4	6-5-5		10-12
	18	12-4	6-4-6	18		5-5-6
	19	6-4-5	5-5-6	4-6		3-9-4-7

day following cessation of treatment and the weights of various organs were obtained. Vaginal smears were obtained from the remaining animals in the next 42 days. The animals were then sacrificed and organ weights were recorded.

The daily oral administration of doses of 1 mg. of MER-25 did not interrupt the cyclical changes in the vaginal epithelium (Table 5); however, completely cornified smears did not occur during this period of drug administration. Increasing the dose to 5 mg. produced smears devoid of cornified cells which lasted until the drug was withdrawn or the animals were sacrificed. Following withdrawal of MER-25, cyclical changes in the vaginal epithelium were established within two to six days in the four remaining animals.

Organ weights for animals sacrificed immediately at the end of the drug period and for those sacrificed 42 days later are summarized in Table 6.

The administration of MER-25 decreased weights of the pituitary, uterus and ovaries. Adrenal weight was not altered by this drug. Forty-two days following withdrawal of MER-25, the organ weights of the treated animals were similar to those of the control animals.

TABLE 6. MEAN BODY WEIGHTS AND ORGAN WEIGHTS OF INTACT FEMALE RATS

Treatment	Body wt., gm. Initial	Body wt., gm. Final	Pituitary, mg.	Adrenals, mg.	Uterus, mg.	Ovaries, mg.
31 days treatment*						
MER-25	123	206	9.1 ± 0.3†	52.5 ± 2.1	169 ± 4	43.7 ± 5.8
Control	126	223	13.1 ± 0.3	56.4 ± 3.1	411 ± 66	57.6 ± 2.9
42 days after treatment						
MER-25	206	229	11.5 ± 0.4	58.0 ± 2.0	426 ± 88	67.4 ± 4.3
Control	223	255	13.7 ± 0.7	55.8 ± 2.0	483 ± 21	60.8 ± 2.3

* 1 mg./day of MER-25 for 14 days followed by 5 mg./day of MER-25 for 17 days.
† All organ weights expressed as Mean ± standard error.

Effect upon Pregnancy. Time of mating of rats was determined by the presence of sperm in daily vaginal smears. MER-25 was administered orally in daily doses of 25 mg./kg. from day one to days 23–25, from day 13 to days 21–26, and from days 4 through 7 following mating. Control animals were administered olive oil in the same manner. Food consumption and body weight changes were recorded. If parturition occurred, the condition of the pups was noted. Animals failing to deliver litters were autopsied at the approximate time for parturition. In the experiment in which MER-25 was administered from days 4 through 7 following mating, the animals were sacrificed on day 12.

Nine of 12 control rats in the study administered placebos from day one of pregnancy, and 9 of 11 control rats in the study administered placebos from day 13 of pregnancy bore normal litters. The day of parturition ranged from 22–25 days with an average of 24 days.

None of 12 rats administered 25 mg./kg./day of MER-25, beginning with the first day after finding sperm in the vagina, bore litters. These rats gained little or no weight as compared to the average weight increase of 59 gm. for the control animals during the gestation period (Fig. 4). The animals administered MER-25 were autopsied 22–25 days following mating. No fetus was found in the uteri. However, hemorrhagic areas, which may have been implantation sites were observed in the uteri of one-half of the animals.

Ten of 11 rats administered MER-25 in daily doses of 25 mg./kg. beginning with day 13 of pregnancy gave birth to young. The day of parturition ranged from 21–26 days with an average of 23 days. Delivery was prolonged, bleeding through the vagina being observed from 2–4 days prior to parturition as compared to one day for the controls. Many of the pups were dead at birth. Several of the rats bore one or more live pups; however, none survived for more than three days. The uterus of one rat which died after bleeding for three days contained 9 well developed pups. The food consumption of all rats administered MER-25, beginning with the 13th day of pregnancy, decreased rather severely (Fig. 4) and the body weight gain was less than that for the controls (Fig. 4). None of the nine rats administeres MER-25 on days 4–7 post mating were pregnant or had implantation sites when autopsied on day 12. Four of the 7 control animals had pregnant uteri. Two of the 3 control rats which were not pregnant had reproductive tract pathology.

Effect upon Lactating Rats and Nursing Pups. Two groups of eight rats each were employed. The litters were reduced to six pups on the day of parturition except for two rats each which had only four pups. One of the groups containing the two mothers with four pups each was administered 25 mg./kg. of MER-25 by stomach tube for 22 days beginning on the day of parturition. The other group served as a control and was administered similar volumes of olive oil. Body weight and food intake of the mothers and body weight gain of the pups were recorded. Male and female offspring of the control and MER-25 groups were mated within their own groups and other offspring of the two groups were cross-mated at an age of 132–145 days.

Daily oral administration of 25 mg./kg. of MER-25 lowered the food in-

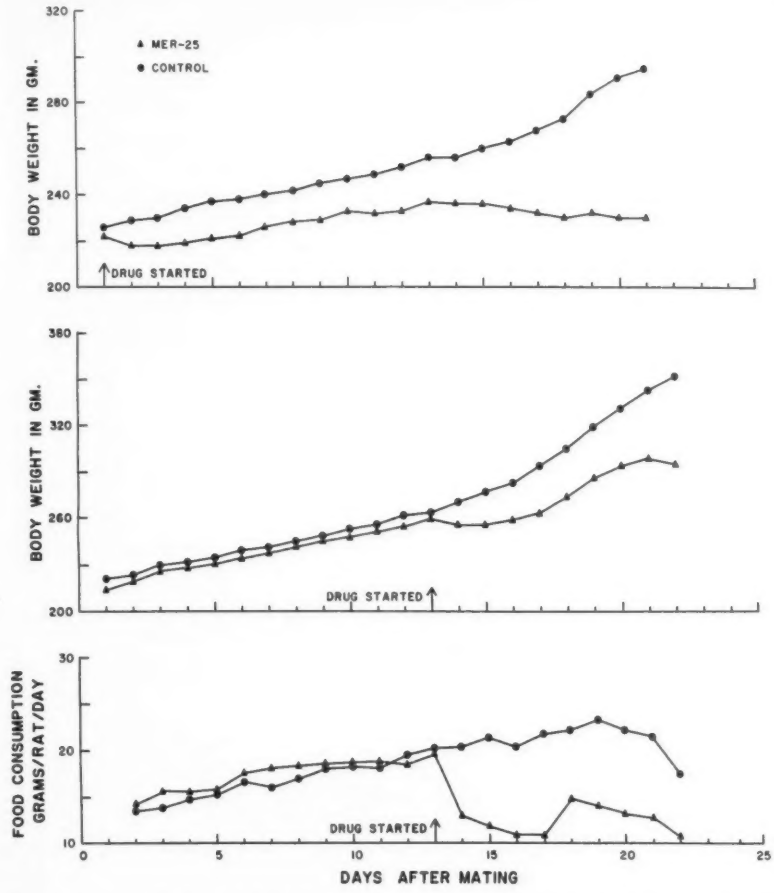


FIG. 4. Upper—Body weight gain of female rats receiving MER-25 (25 mg./kg./day, orally) starting on the first day post mating.

Middle and lower—Body weight gain and food consumption of female rats receiving MER-25 (25 mg./kg./day, orally) starting on day 13 post mating.

take as compared to controls (Fig. 5). The body weight of the mothers first decreased but after five days increased to that of the controls and reached the average control weight by the 16th day, even though the food intake remained at a lower level. The pups of the mothers administered MER-25 gained less weight than the pups of the control animals. It is not known if this is a direct effect of MER-25 on lactation or if it is the result of the lowered food intake of the mothers, or if the drug or a metabolite is passing to the pups through the milk.

During the experiment, there were nine deaths in litters of MER-25-

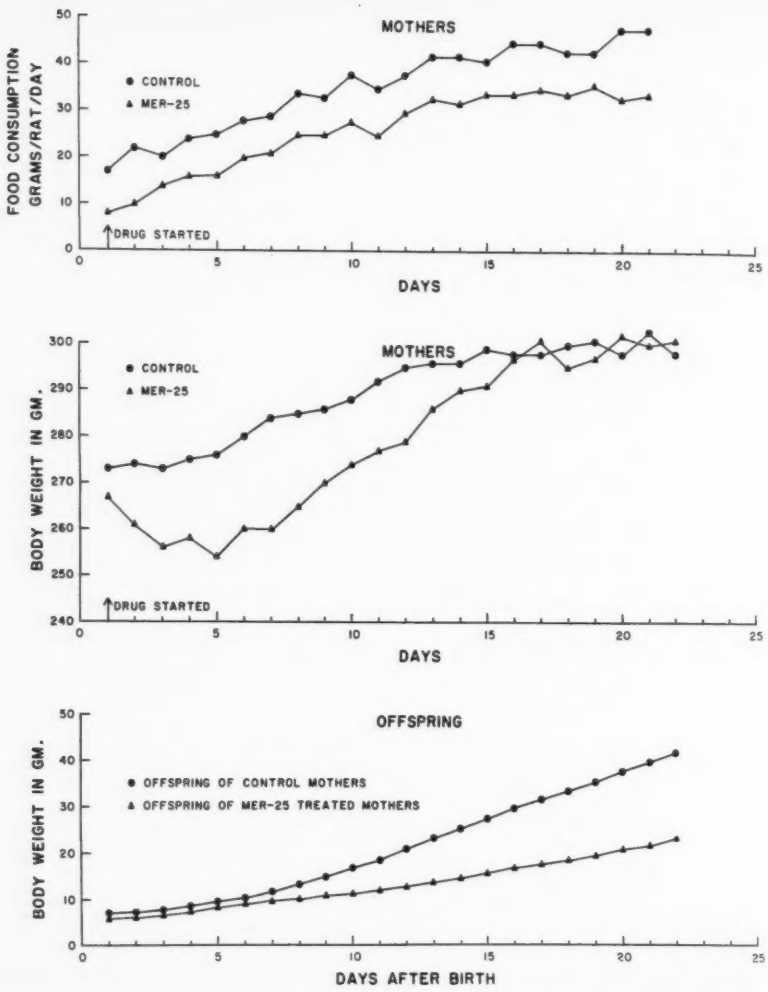


FIG. 5. Upper and middle—Food consumption and body weight gain of lactating rats receiving MER-25 (25 mg./kg./day, orally) starting on the day of parturition.

Lower—Body weight gain of nursing offspring of female rats receiving MER-25 (25 mg./kg./day, orally) starting on the day of parturition.

treated mothers, and eight deaths in the litters of the control animals.

At the approximate age of 140 days the male and female offspring of control mothers weighed 10–20% more than the corresponding offspring of MER-25-treated mothers. Matings of control female offspring to control male offspring or to male offspring of MER-25-treated mothers resulted in pregnancy and normal parturition in 8 of the 9 rats. The ninth female

died on the 23rd day of gestation and well formed pups were found *in utero*.

There were no pregnancies among the 14 female offspring of MER-25-treated mothers although all of these animals copulated. Three of these rats had one or more apparent implantation sites.

Uterine Effects in Immature Rabbits. The McPhail technique (23) was employed to determine progestational and anti-progestational activities. Dose groups of five animals each were employed. The rabbits were administered three intramuscular injections of 5 μg. of estradiol on alternate days. They then received five daily intramuscular injections of progesterone, MER-25 and a combination of progesterone and MER-25.

The McPhail method was also employed to determine if MER-25 would sensitize the uterus to progesterone and if it would inhibit the estrogenic sensitization of the uterus to progesterone. Dose groups of six immature rabbits were injected daily intramuscularly with 0.15 mg. of progesterone for five days starting on the seventh day of the study. Two groups were primed with total doses of 5 μg. of estradiol I.M., and two were primed with total doses of 15 μg. of estradiol I.M. This estrogen was administered on days 1, 3 and 5 of the study. Two groups received no estradiol at all. One of these served as a control and was given olive oil in place of the estrogen and the second group was administered 100 mg. of MER-25 daily for the first six days of the experiment. One of the groups receiving the 5 μg. dose of estradiol and one of the groups receiving the 15 μg. dose of estradiol were injected I.M. with 100 mg. of MER-25 per day for eleven days starting with the first day of the experiment.

Uterine weight and histology of rabbits receiving a total dose of 500 mg. of MER-25 demonstrate that this compound does not have progestational activity and does not inhibit the progestational activity of progesterone (Table 7).

TABLE 7. PROGESTATIONAL AND ANTI-PROGESTATIONAL ACTIVITY IN RABBITS

Pre-treatment (priming)—total dose	Treatment—total dose	Uterine wt., gm.	Uterine histology*
—	—	0.26	4N
—	Progesterone, 0.75 mg.	0.29	2L, 1N
Estradiol, 15 μg.	—	1.53	11L
Estradiol, 15 μg.	Progesterone, 0.75 mg.	2.93	14H
Estradiol, 15 μg.	MER-25, 500 mg.	1.42	5L
Estradiol, 15 μg.	Progesterone, 0.75 mg. + MER-25, 500 mg.	3.25	5H

* H—Typical progestational uterus.
M—High columnar type endometrium with primary invagination; moderate glandular proliferation; diffuse stroma.
L—Thickening of endometrium but only slight invagination; "flattened glands" (narrow lumen); stroma compact. This type exemplified by estrogenic stimulation.
N—No stimulation—control type—non-primed.
Number preceding letter indicates number of animals.

MER-25 for practical purposes was ineffective as a uterine priming agent in total doses of 1100 mg. (Table 8). This amount of MER-25 was large enough to block the uterine priming activity of 5 μg. of estradiol and was able in 50% of the animals to prevent partially the uterine priming action of 15 μg. of estradiol.

TABLE 8. UTERINE SENSITIZATION AND ESTROGEN ANTAGONISM IN RABBITS

Treatment*	Uterine wt., gm.	Uterine histology†
Control	0.16	6N
Estradiol, 5μg. on days 1, 3, and 5	2.51	5H
Estradiol, 1.67 μg. on days 1, 3, and 5	2.03	5H, 1M
MER-25, 100 mg. on days 1 through 11	0.58	1M, 2L, 3N
MER-25, 100 mg. on days 1 through 11 Estradiol, 5 μg. on days 1, 3, and 5	2.07	3H, 3M
MER-25, 100 mg. on days 1 through 11 Estradiol, 1.67 μg. on days 1, 3, and 5	0.83	3M, 1L, 2N

* All groups administered 0.15 mg. progesterone on days 7 through 11.
† See footnote Table 7.

Effects in Baby Chicks. Groups of eight seven-day old chicks were injected subcutaneously twice daily for six days with MER-25, estradiol and a combination of both compounds. The birds were sacrificed on the seventh day, and the oviducts were weighed and the plasma phospholipid levels were determined (24, 25).

Estradiol elevated the level of plasma phospholipids by 100% and the weights of the oviducts by approximately 1500%. MER-25 by itself had no effect upon phospholipids or oviduct weight. The compound, however, completely prevented the estrogen induced phospholipid elevation and inhibited approximately 65% of the estrogen induced oviduct weight increase. The daily dose of 3 mg. of MER-25 was as effective as the daily dose of 30 mg. in preventing the effects of 0.1 mg. of estradiol (Table 9).

Effect upon Intact Male Rats. MER-25 was administered once a day by stomach tube to young mature rats alone and in combination with estradiol. Dose groups of ten animals each were employed. Estradiol was administered subcutaneously once a day in doses of 10 Rat Units (3.5 μg.). In the first experiment, MER-25 was administered in daily doses of 10 mg. and in the second experiment, it was administered in daily doses of 1.0 and 0.2 mg. The animals were sacrificed after 31–33 days of drug administration. The pituitaries were removed, weighed, mascerated and suspended in water in preparation for gonadotrophin determinations. The pituitaries from animals in each dose group in the second experiment were pooled before macerating. In the first experiment, the pituitaries were not pooled. Twenty-one-day-old rats were employed for the gonadotrophin assays. Each assay animal was administered subcutaneously either one pituitary or the equivalent of one pituitary in five or six doses, two doses per day for two days, and

TABLE 9. ESTROGEN ANTAGONISM IN CHICKS

Treatment	Daily dose	Body wt., gm.		Oviduct, mg.	Plasma phospholipids, mg.%
		Initial	Final		
Control	—	56	93	11	237
Estradiol	0.1 mg.	51	84	169	458
MER-25	3 mg.	54	88	11	252
MER-25	30 mg.	57	72	8	247
MER-25 3 mg. Estradiol 0.1 mg.		51	80	61	279
MER-25 30 mg. Estradiol 0.1 mg.		52	63	61	240

either one or two doses on the third day. The rats were sacrificed 96 hours after the first dose, and the uteri and ovaries were removed and weighed.

Pituitary sections from rats administered MER-25 in doses of 10 mg. and from rats administered 10 R.U. estradiol were stained with Gomori's aldehyde-fuchsin (26) and by the periodic acid-Schiff method.

Animals administered 10 R.U. of estradiol per day gained less weight than the control animals. MER-25, in doses of 10 mg. per day, decreased the rate of body weight gain, but the decrease was considerably less than that caused by estradiol. Body weight gain for rats administered 1.0 and 0.2 mg. of MER-25 daily was similar to that of the control animals.

The daily administration of estradiol, as is typical with estrogens, increased the weights of the adrenals and pituitaries and decreased weights of the seminal vesicles, ventral prostate and testes. This lowering in weight of the reproductive organs by estrogens results from a decreased amount of pituitary gonadotrophins (29). The ovarian and uterine weights of assay animals administered the equivalent of one pituitary from the estradiol-treated animals were similar to those of the saline recipient controls.

MER-25 did not alter the weights of the adrenals of the rats. Slight changes occurred in the pituitary weights but this may be related to changes in body weight. The reproductive organs of rats administered daily doses of 10 mg. and 1 mg. of MER-25 weighed less than those of the controls. The effect on the reproductive organs, however, was much smaller than that of estradiol. MER-25 in doses of 0.2 mg. per day did not alter organ weights. Even though the weights of the reproductive organs of the animals administered doses of 1 mg. and 10 mg. of MER-25 were slightly less than those of the controls, the gonadotrophin content of the pituitaries was similar to that of the control animals.

The administration of MER-25 in daily oral doses of 10 mg. and 1 mg. in combination with estradiol prevented the estrogen-induced pituitary hypertrophy. It did not alter the response of the adrenals and the reproductive organs to the estrogen nor did it prevent lowering of pituitary gonadotrophins. The 0.2 mg. daily doses of MER-25 had no effect upon estradiol-induced changes in the rat (Table 10).

TABLE 10. INTACT MALE RATS TREATED FOR 31 TO 33 DAYS

Treatment	Daily dose	Body wt., gm.		No. of rats	Mean organ weights					Assay of pituitaries	
		Initial	Final		Adrenals mg.	Seminal vesicles, mg.	Ventral prostate, mg.	Testes, mg.	Pituitary, mg.	Ovaries, mg.	Uterus, mg.
Control		149	286	20	37.3	244	324	3201	9.1	83	94
Estradiol	3.5 μg.	150	211	20	52.4	36	20	1033	14.5	15	36
MER-25	10 mg.	144	243	8	38.2	155	133	2992	7.2	81	96
MER-25	1 mg.	154	284	9	37.8	212	249	2999	8.7	78	103
MER-25	0.2 mg.	155	294	10	36.9	263	349	3318	9.4	83	93
MER-25 + Estradiol	10 mg. + 3.5 μg.	147	214	9	51.2	22	17	904	7.6	17	62
MER-25 + Estradiol	1 mg. + 3.5 μg.	154	199	9	44.8	29	15	670	10.4	13	33
MER-25 + Estradiol	0.2 mg. + 3.5 μg.	155	213	10	48.8	39	23	984	14.9	13	42
Recipient controls (saline injected)										14	36

Even though MER-25 did not prevent the lowering of pituitary gonadotrophins by estradiol, the cytology of the pituitaries from animals administered 10 mg. doses of MER-25 in combination with 10 R.U. of estradiol could not be differentiated from those of normal animals (Fig. 6 and 7). The gonadotrophs were well granulated and appeared normal. In con-

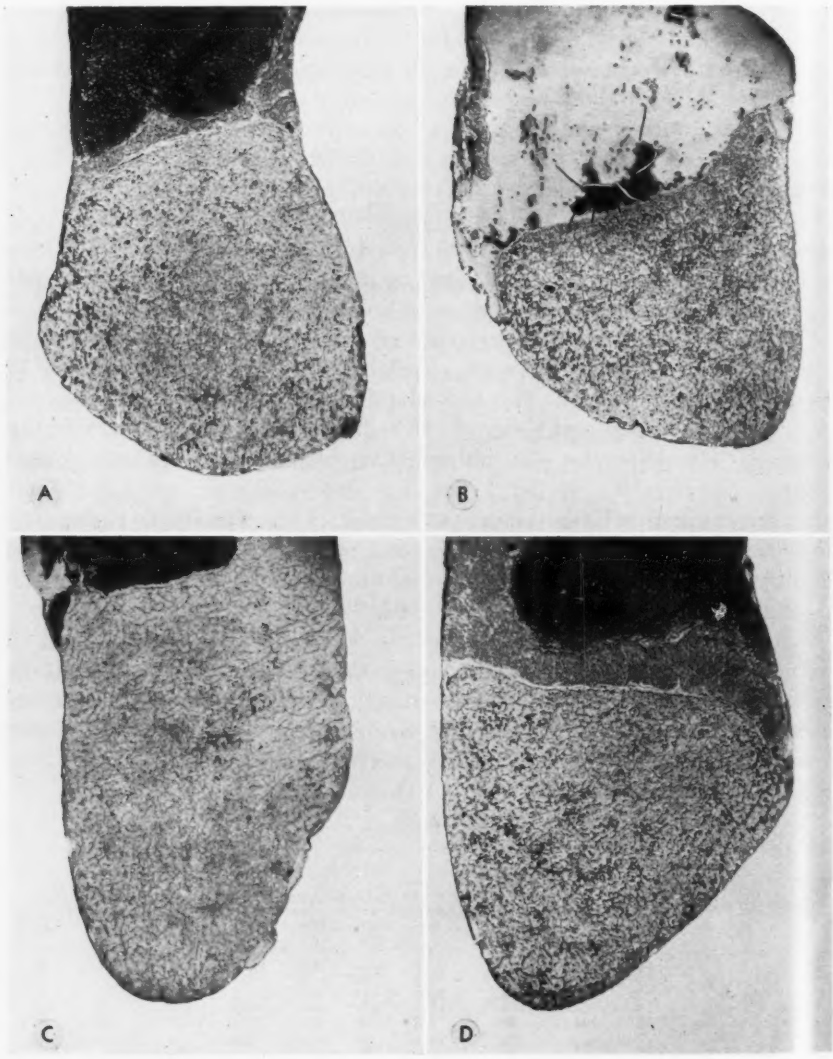


FIG. 6. Rat pituitaries—aldehyde-fuchsin (Gomori) ×30. (A) Terminal control, (B) MER-25, 10 mg./day, (C) Estradiol, 3.5 μg./day, (D) MER-25, 10 mg./day +Estradiol, 3.5 μg./day.

trast to this, the pituitary gonadotrophs of animals administered estradiol alone were completely degranulated (Fig. 7C).

Effect of Castrate Male Rats. Rats were castrated on the day before the start of drug administration. Ten animals were administered 10 mg./day of MER-25 by the drug diet

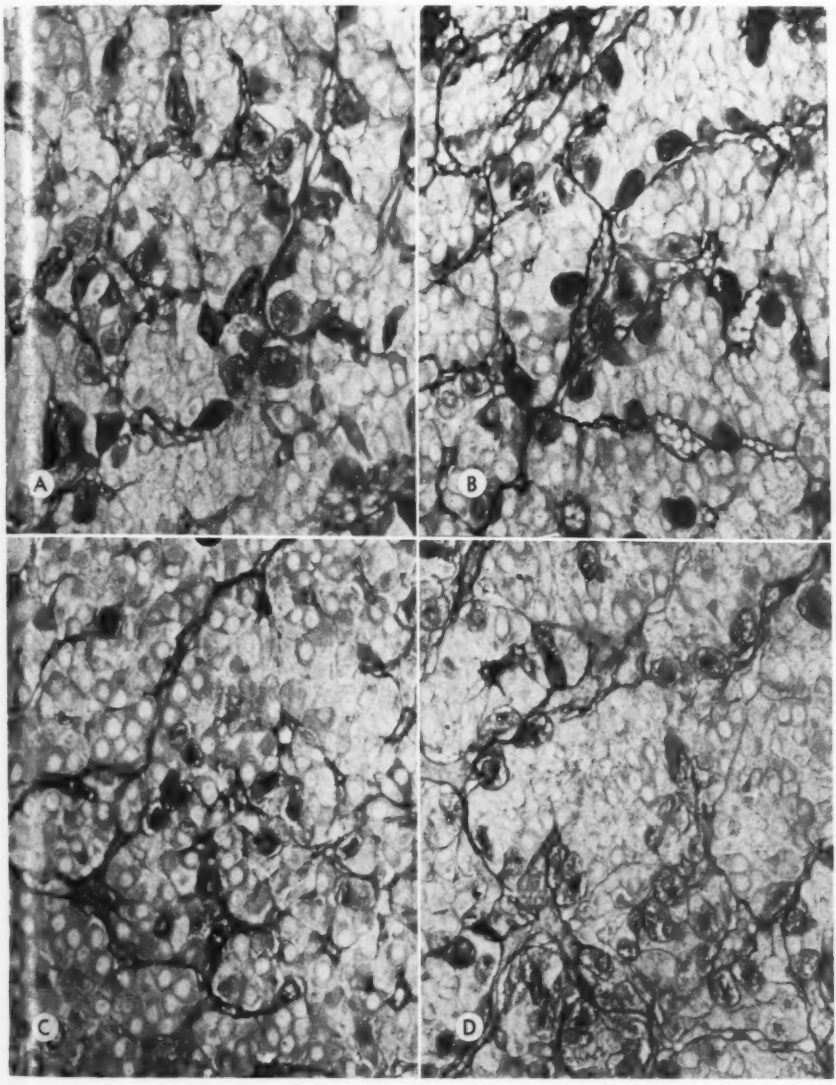


FIG. 7. Rat pituitaries—aidehyde-fuchsin (Gomori) and PAS ×400. (A) Terminal control, (B) MER-25, 10 mg./day, (C) Estradiol, 3.5 μg./day, (D) MER-25, 10 mg./day + Estradiol, 3.5 μg./day.

TABLE 11. MALE RATS TREATED FOR 30 DAYS

Treatment	Body wt., gm.		Pituitary, mg.	Adrenals, mg.	Seminal vesicles, mg.	Ventral prostate, mg.
	Initial	Final				
Intact-control	145	260	7.4 ± 0.2*	32.7 ± 0.9	248.0	326.0
Castrate-control	138	255	12.6 ± 0.4	41.2 ± 1.3	18.2	14.9
Castrate-MER-25, 10 mg./day	144	192	8.6 ± 0.4	43.2 ± 1.3	19.0	14.2

* Mean ± standard error.

method. A similar castrate group and an intact control group were fed a standard diet. The animals were sacrificed after thirty days of treatment, and body and organ weights were recorded.

Groups of 10 immature, castrate, male rats were administered 50 and 100 mg./kg./day of MER-25 subcutaneously alone or in combination with 1 mg./kg./day of testosterone propionate in olive oil for 7 days. Control groups received testosterone propionate or olive oil. The animals were sacrificed on the eighth day and the seminal vesicles, and ventral prostate were removed and weighed.

MER-25 (10 mg./day) decreased body weight gain of the rats (Table 11). This dose did not prevent the decrease in weight of the seminal vesicles and ventral prostate which follow castration. MER-25, however, did prevent the castration hypertrophy of the pituitaries. MER-25 is not androgenic as evidenced by its failure to increase the seminal vesicle and ventral prostate weight in the castrate animal (Table 11). MER-25 in subcutaneous doses up to 100 mg./kg./day did not antagonize the seminal vesicle and ventral prostate weight gain, induced by 1 mg./kg./day of testosterone propionate.

Activity in Parabiotic Female Rats. The gonadotrophin inhibitory activity of MER-25 was compared to that of estradiol in parabiotic, immature female litter mate rats by the method of Byrnes and Meyer (27). MER-25 or estradiol was injected subcutaneously for ten days to the ovariectomized parabiotic partner. The animals were sacrificed on the eleventh day, and the ovaries and the uteri were removed and weighed.

MER-25 is a weak gonadotrophin inhibitor as indicated by the ovarian and uterine weights of the intact partner (Table 12). The pituitary gonadotrophins of the castrate partner were inhibited by daily doses of 10 mg.

TABLE 12. GONADOTROPHIN INHIBITION IN PARABIOTIC RATS

Treatment	Daily dose	No. of pairs	Body wt., gm.		Intact partner		Ovariectomized partner uterus, mg.
			Initial	Final	Ovaries, mg.	Uterus, mg.	
Control	—	24	155	198	120	143	37
MER-25	10 mg.	5	165	216	36	74	47
MER-25	1 mg.	8	160	214	63	128	49
MER-25	0.1 mg.	5	160	210	127	143	54
Estradiol	0.1 μg.	3	—	212	26	75	122
Estradiol	0.015 μg.	5	155	193	32	142	46
Estradiol	0.005 μg.	2	145	187	20	95	26
Estradiol	0.001 μg.	4	150	199	56	121	41

and partially inhibited by daily doses of 1 mg. Comparable doses of estradiol to produce complete and partial inhibition of the gonadotrophins are 0.005 μg. and 0.001 μg., respectively.

Acute Toxicity in Mice. Mice weighing 14–20 gm. were employed. MER-25 was administered orally and subcutaneously, and an aqueous solution of the corresponding citrate salt was administered intravenously. The animals were under observation up to seven days post injection.

Subcutaneous doses in oil up to 3000 mg./kg. did not cause any signs of toxicity during a seven day observation period. In oil suspension, the acute oral LD_{50} was 1700 ± 130 mg./kg. With the citrate salt administered intravenously in saline, the LD_{50} was 105 ± 15 mg./kg. Deaths occurred within 48 hours following oral administration, and from 2–20 minutes following intravenous administration. Prior to death, the animals were depressed.

Subchronic Toxicity Study in Rats. Eight groups of eight female rats and eight groups of eight male rats weighing 125–140 gm. each were employed. Two groups of males and two groups of females served as controls. One group of male controls and one group of female controls were administered olive oil by stomach tube and the other control groups were administered olive oil subcutaneously in volumes similar to those employed for drug administration. The other groups of animals were administered MER-25 orally and subcutaneously in total doses of 5, 25 and 125 mg./kg. The total daily oral doses were administered in two divided doses per day, and the subcutaneous doses were administered once a day. The animals were dosed five days a week, and with the exception of the 125 mg./kg. subcutaneous dose groups, the period of drug administration was six weeks. The 125 mg./kg. subcutaneous groups were sacrificed after four weeks of drug administration because of severe irritation of the injection sites. All animals were sacrificed within six days following the last administered dose. Blood counts were obtained prior to starting drug administration and again during the last week of drug administration. Body weight changes and food consumption were recorded. Organ weights were determined at autopsy, and various tissues were examined histologically.

Body weight changes and food consumption for male and female animals administered daily 5 mg./kg. and 25 mg./kg. of MER-25 orally and subcutaneously were similar to those of their respective controls. The oral and subcutaneous administration of daily doses of 125 mg./kg. decreased food intake, and the rate of body weight increase was considerably less than that of the controls or other experimental groups.

The total leucocyte count increased in the male rats administered MER-25 subcutaneously at all doses and in the female rats administered 125 mg./kg./day subcutaneously. The highest subcutaneous dose also increased the per cent of granulocytes in both the male and female animals. These increases probably resulted from the local irritant action of this drug. The total leucocyte count and the per cent granulocytes were also elevated in the female animals administered the highest dose of MER-25 orally. This effect was possibly related to the lipoid pneumonia prevalent in this group. There were no other significant alterations in the hematology.

Oral and subcutaneous administration of MER-25 decreased the weight of the testes, seminal vesicles and ventral prostates of the males, the uteri of the females, and the pituitaries and thymus of the males and females. The oral administration had a greater effect than did the subcutaneous administration, and the larger the dose of MER-25, the greater the depression of these organ weights.

The livers from male and female animals administered daily oral doses of 125 mg./kg. were slightly heavier than those from the control animals or from the lower dose group animals. This effect was more pronounced in the female rats. Histologically these enlarged livers had minimal fatty vacuolization.

With the exception of the livers, histological studies were made only on the tissues of animals administered MER-25 orally.

Spermatogenesis was absent in the testes of two of the eight animals administered 125 mg./kg./day and was greatly depressed in a third animal.

TABLE 13. PER CENT OF ANIMALS WITH OPEN VAGINA

Treatment	Daily dose, mg./kg.	Days after birth																				
		30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Control	—	10	10	10	10	10	10	10	10	10	30	60	70	70	80	80	80	80	90	90	90	100
MER-25	50						20	20	30	30	60	70	100									
MER-25	5				20	70	90	90	90	90	100											

The seminal vesicles and ventral prostates had varying degrees of secretory activity and atrophic changes. These changes were most pronounced in the two animals having no spermatogenesis.

The vaginae of all rats administered MER-25 had various degrees of mucification of the epithelium. The ovaries of three of the four surviving female rats administered 125 mg./kg./day of MER-25 orally were smaller than those of the control animals. Histologically these ovaries contained a greatly reduced number of follicles. Ovarian histology in all the other drug-treated animals was similar to that of the control animals.

No significant gross or histological alterations were observed in the adrenals, thyroids, pancreas, kidneys, heart, aorta, lungs, spleen, esophagus, stomach, large and small intestines, and urinary bladder.

Effect on Vaginal Opening in Immature Rats. The finding that MER-25 produced vaginal mucification in rats made it of interest to determine its effect on vaginal opening in immature animals.

Three groups of ten immature female rats weighing approximately 50 gm. were employed. Two of the groups were administered MER-25 by the drug diet method in daily doses of approximately 5 mg./kg. and 50 mg./kg. The third group served as a control and received the stock diet. The animals were examined daily for vaginal opening.

The mean day of vaginal opening was day 35 for the 5 mg./kg./day group, day 39 for the 50 mg./kg./day group, and day 41 for the control (Table 13).

Effects in Castrate Female Monkeys. Two castrate female monkeys (Macaca mulatta) weighing approximately 4 kg. each were employed. Vaginal smears were made daily and read according to the method of Shorr (28). Estradiol was administered subcutaneously and MER-25 was administered orally in hard gelatin capsules. A series of three experiments were performed. In the first of these, the dose of the estrogen was 25 μg./kg./day, and in the last two studies, the dose was 10 μg./day. The doses of MER-25 employed ranged from 25 mg./kg./day to 150 mg./kg./day. A continuous record of vaginal cornification, sex skin swelling and reddening, and withdrawal bleeding was kept.

The protocols of the experiments and the experimental results are summarized in Figure 8. The administration of estradiol in doses of 25 μg./kg./

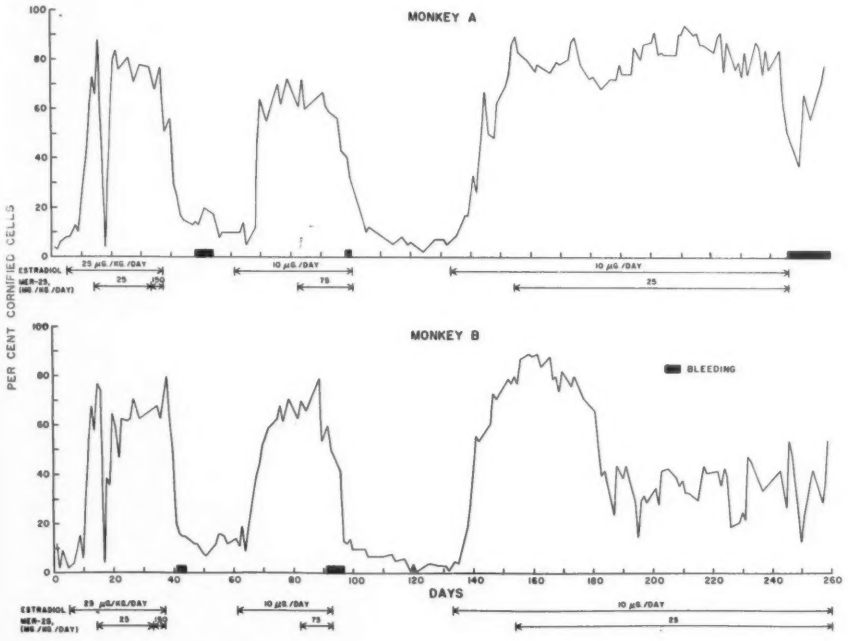


FIG. 8. Effect of MER-25 on vaginal cornification and withdrawal bleeding in castrate monkeys maintained on estradiol.

day in the first experiment and in doses of 10 μg./animal/day in the next two experiments increased the percentage of cornified cells and produced reddening and swelling of the sex skin.

Experiment 1. MER-25, at 30 mg./kg./day administered together with estradiol (25 μg./kg./day) produced a transient fall in the per cent of cornified cells to the castrate level and some lessening of the reddening and swelling of the sex skin. The administration of 150 mg./kg./day of MER-25 for four days stopped the animals from eating; therefore, the drugs were

discontinued. Withdrawal bleeding occurred four days following the discontinuance of the administration of estradiol and MER-25.

Experiment 2. The per cent of estradiol-induced cornified cells (10 μg./animal/day) decreased on the seventh day of MER-25 administration (75 mg./kg./day) in one monkey (B) and on the fourteenth day in the second monkey (A). Withdrawal bleeding occurred on the ninth day of MER-25 administration in monkey B, and on the sixteenth day in monkey A.

Experiment 3. The per cent of estradiol-induced cornified cells (10 μg./animal/day) in monkey B decreased from approximately 90% to approximately 50% after 35 days of administration of 25 mg./kg. of MER-25 and remained at that level for the remainder of the experiment. Uterine bleeding occurred five days after withdrawal of the drug and estrogen treatment. In the second monkey (A), uterine bleeding, accompanied by a rapid decrease in cornified cells, occurred on the 92nd day of MER-25 administration.

Chronic Toxicity in Intact Monkeys. MER-25 in doses ranging from 1 mg./kg./day to 60 mg./kg. twice a day was administered orally in hard gelatin capsules to two mature female monkeys and one male monkey for approximately seventy weeks. Two male and two female monkeys were administered sucrose filled placebo capsules and served as controls. Except for the first two dose periods, MER-25 was administered twice a day, five days a week and once a day during the weekends. During the first two dose periods, MER-25 was administered once a day, six days a week. Body weight changes and food consumption was recorded regularly. Blood counts were obtained just prior to starting the drug administration and at monthly intervals during the periods of the treatment. The length of the menstrual cycles were noted, as well as changes in the per cent of cornified vaginal cells.

The daily administration of MER-25 in doses up to 40 mg./kg. twice a day did not alter the body weights of the three animals, with the exception of one female monkey. In this latter animal, after 42 days of treatment, the drug was discontinued for a period of 35 days. Drug administration was resumed after this rest period. The administration of doses of 60 mg./kg. twice a day for ten days decreased food intake of the three animals and caused weight loss. Drug administration was, therefore, discontinued for nine days. The animals, after the nine day rest period, were administered 40 mg./kg. of the drug for 46 days without any signs of toxicity.

MER-25 did not cause any changes in hematological values of these animals.

The length of the menstrual cycles of the female monkeys are summarized in Figure 9. MER-25, in doses of 1 mg./kg./day to 10 mg./kg. twice a day, appeared to have lengthened the cycles. Within two weeks after increasing the dose of MER-25 to 40 mg./kg. twice a day, menstruation occurred in both monkeys. Thereafter, no menstruation occurred for 167 days in one animal and 197 days in the second animal. This latter monkey was sacrificed at that time. Daily doses up to 20 mg./kg. twice a day did not

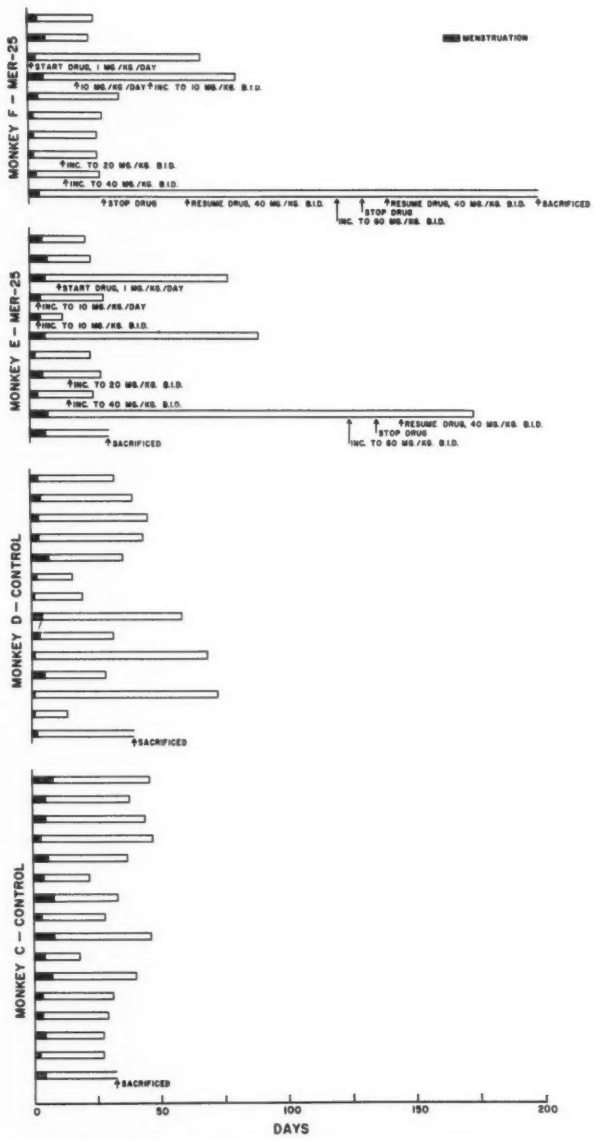


FIG. 9. Effect of MER-25 on the length of consecutive menstrual cycles in intact monkeys.

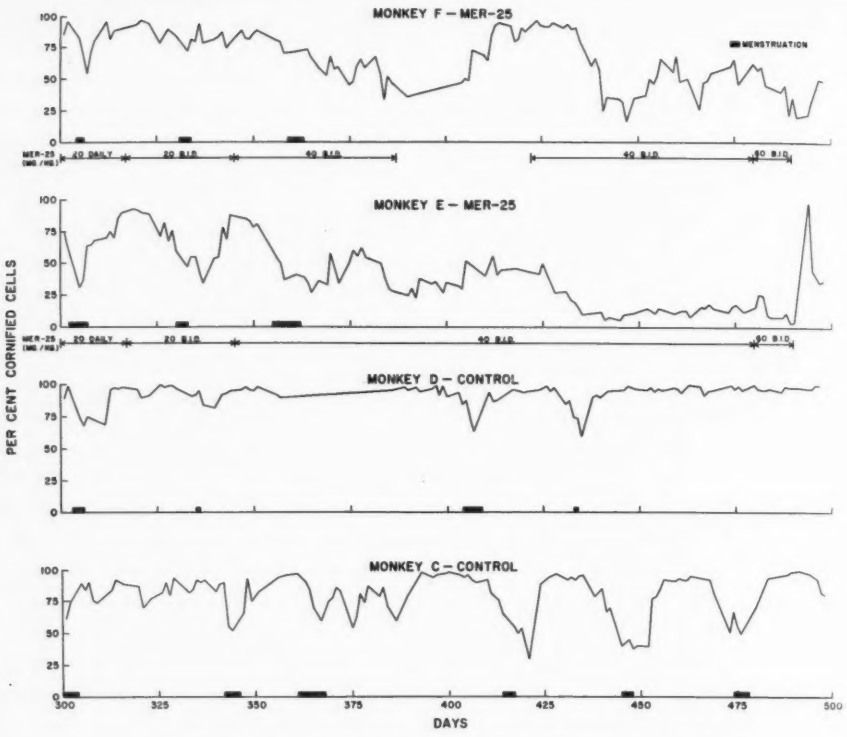


FIG. 10. Effect of MER-25 upon the per cent of vaginal cornified cells in intact monkeys.

alter the per cent of cornified cells in the vaginal smears. The per cent of cornified cells, beginning with day 300 of the experiment, are plotted in Figure 10. For the first 40 days shown on the graph the vaginal smears remained typical of the animals during the early part of the experiment. The daily administration of doses of 40 mg./kg. twice a day decreased the per cent of cornified cells. This effect is more pronounced in monkey E than in monkey F.

DISCUSSION

The experiments reported demonstrate that MER-25 is an estrogen antagonist in the mouse, rat, rabbit, monkey and chick. The antagonism is not limited to inhibiting the hormonal activity of natural occurring steroids since the effects of synthetic non-steroidal estrogens are also counteracted. MER-25, unlike the previously reported antiestrogenic steroids, completely inhibits estrogen-induced uterine growth. It inhibits the effect of estradiol on the vagina, pituitary, and chick oviduct. The marked rise i

the plasma phospholipids of the chick following estrogen administration is prevented by MER-25. The antagonism of estrogens is not produced through effects on the ovary since the anti-hormonal activity is present in the castrated as well as the intact animal.

Weak gonadotrophin inhibition is the only other effect demonstrated by this compound.

MER-25 prevents estradiol induced hypophyseal hypertrophy. Similar results were reported for chlorotrianisene (TACE), (30). However, MER-25, unlike chlorotrianisene, does not produce degranulation of the gonadotrophs, and MER-25 prevents the estradiol or chlorotrianisene-induced degranulation and disappearance of the hypophyseal gonadotrophs (31).

MER-25 administration to lactating rats resulted in infertile female offspring but fertile male offspring. A possible explanation of this is that this compound affects the germinal epithelium adversely, acting either directly or indirectly. This is consistent with the hypothesis that all of the oocytes present in the mature ovary are present at birth while spermatogenesis occurs throughout the reproductive life of the male (32). This compound, based on the findings reported, may be useful in elucidating the mechanisms of action of gonadotrophins and estrogen.

Acknowledgments

The authors are grateful to Dr. Roy O. Greep for the cytological study of the male rat pituitaries; to Mr. James Knox Smith for the toxicological studies in rats, mice and monkeys; and to Dr. Thomas R. Blohm for the study in baby chicks.

REFERENCES

1. Hisaw, F. L., J. T. Velardo and C. M. Goolsby: *J. Clin. Endocrinol. and Metab.* **14:** 1134. 1954.
2. Velardo, J. T. and S. H. Sturgis: *Proc. Soc. Exper. Biol. and Med.* **90:** 609. 1955.
3. Edgren, R. A. and D. W. Calhoun: *Proc. Soc. Exper. Biol. and Med.* **94:** 537. 1957.
4. Szego, C. M. and S. Roberts: *Am. J. Physiol.* **152:** 131. 1948.
5. Astwood, E. B.: *Am. J. Physiol.* **129:** 302. 1940.
6. Robson, J. M.: *J. Physiol.* **92:** 371. 1938.
7. Huggins, C. and E. V. Jensen: *J. Exper. Med.* **102:** 347. 1955.
8. Mardones, E., R. Inglesias and A. Lipschutz: *Nature* **174:** 839. 1954.
9. Velardo, J. T., F. L. Hisaw and A. F. Bever: *Anat. Rec.* **117:** 552. 1955.
10. Velardo, J. T.: *Am. J. Physiol.* **186:** 468. 1956.
11. Velardo, J. T. and S. R. Sturgis: *Am. J. Physiol.* **183:** 259. 1955.
12. Roberts, S. and C. M. Szego: *J. Biol. Chem.* **201:** 21. 1953.
13. Szego, C. M.: *Endocrinology* **50:** 429. 1952.
14. Beyler, A. L. and C. M. Szego: *Endocrinology* **54:** 334. 1954.
15. Robson, J. M.: *J. Physiol.* **96:** 21P. 1939.
16. Talalay, P., M. M. Dobson, C. M. Ebersole and C. Huggins: *Endocrinology* **50:** 574. 1952.
17. Velardo, J. T.: *Anat. Rec.* **122:** 478. 1955.
18. Velardo, J. T. and S. H. Sturgis: *J. Clin. Endocrinol. and Met.* **16:** 496. 1956
19. Cheymol, J., H. Desarmenien and J. M. Durey: *Therapie* **9:** 353. 1954.

20. HERTZ, R. AND W. H. SEBRELL: *Science* **100:** 293. 1944.
21. DAVIS, J. S.: *Proc. Soc. Exper. Biol. and Med.* **95:** 247. 1957.
22. DAVIS, J. S., R. K. MEYER AND W. H. MCSHAN: *Endocrinology* **59:** 505. 1956.
23. MCPHAIL, M. K.: *J. Physiol.* **83:** 145. 1935.
24. FISKE, C. H. AND Y. SUBBAROW: *J. Biol. Chem.* **66:** 375. 1925.
25. YOUNGBERG, G. E. AND M. V. YOUNGBERG: *J. Lab. and Clin. Med.* **16:** 158. 1930.
26. GOMORI, G.: *Am. J. Clin. Path.* **20:** 665. 1950.
27. BYRNES, W. W. AND R. K. MEYER: *Endocrinology* **48:** 133. 1951.
28. SHORR, E.: *Science* **94:** 545. 1941.
29. GREEP, R. O. AND I. O. JONES: *Recent Progress in Hormone Research* **5:** 197. 1950.
30. SEGAL, S. J. AND C. R. THOMPSON: *Proc. Soc. Exper. Biol. and Med.* **91:** 623. 1956.
31. GREEP, R. O.: Personal Communication. 1957.
32. ZUCKERMAN, S.: *Recent Progress in Hormone Research* **6:** 63. 1951.

INDEPENDENCE OF LH AND ACTH RELEASE FROM THE RAT PITUITARY

NEENA B. SCHWARTZ[1] AND LOIS S. BOSWELL

Department of Physiology, University of Illinois College of Medicine, Chicago, Illinois

ABSTRACT

Cyclic rats known to release LH between 2:00 P.M. and 4:00 P.M. on the day of proestrus were tested for simultaneous ACTH release by measurement of adrenal ascorbic acid concentration between 3:30 and 4:30 P.M. No depletion of ascorbic acid occurred, indicating that ACTH is not released simultaneously with LH.

Pentobarbital treatment at 2:00 P.M. did not cause significant adrenal ascorbic acid depletion in rats of different cycle stages. Therefore, the ability of pentobarbital at 2:00 P.M. to block LH release in proestrous rats is not the result of a non-specific "stressing" action as has been suggested in the literature.

THE question of independence of release of the several anterior pituitary hormones has not been thoroughly investigated (1). Saxton and Greene (2) found a decrease in pituitary TSH and ACTH *content*, as well as gonadotrophin content, following coitus in the rabbit. Cyclic changes in adrenal weight (3) and thyroid I^{131} uptake (4) have been reported during the rat non-pregnant estrous cycle. However, Brown-Grant (5) measuring the release of thyroidal I^{131} found no differences associated with coitus in the rabbit or the stage of the estrous cycle in the rat. "Stress" increases ACTH secretion while decreasing gonadotrophin (6) or TSH (7) secretion. On the other hand, increased gonadotrophin secretion during early pregnancy and pseudopregnancy does not interfere with ACTH release induced by unilateral adrenalectomy (8).

Everett and coworkers (9) have demonstrated that, in four-day cyclic rats exposed to light between 5 A.M. and 7 P.M. pituitary LH release is stimulated between 2:00 and 4:00 P.M. on the day of proestrus. This provides an excellent experimental tool for testing the hypothesis that other hormones might be released obligatorily when one hormone is secreted acutely. In the following experiment, adrenal ascorbic acid concentration was measured in the morning and afternoon in proestrous rats as an endogenous assay for possible ACTH release (10) accompanying the secretion of LH. No evidence of ACTH release was obtained.

The experiment was then repeated using animals injected with pento-

Received February 10, 1958.

[1] Present Address: Institute for Psychosomatic and Psychiatric Research and Training, Michael Reese Hospital, Chicago, Illinois.

barbital at 2:00 P.M. to block LH release in order to ascertain if the pentobarbital simultaneously caused ACTH release as a test of the hypothesis of Dordoni and Timiras (9, 11) that blocking agents, acting as non-specific stressors, prevent LH secretion by eliciting ACTH release. Since significant ACTH release did not occur, the hypothesis must be rejected.

MATERIALS AND METHODS

Virgin female rats (90 days old) obtained in seven lots from Sprague-Dawley, Inc., were housed either one or two per cage in a temperature-controlled room, artificially lighted from 5:00 A.M. to 7:00 P.M. Vaginal smears were taken each morning, and every rat used in these experiments had run at least three consecutive four-day cycles before the cycle during which it was autopsied. In preliminary tests it was confirmed that ovulation was occurring during the night between proestrus and estrus, and that pentobarbital (3 mg./100 g.) (9) at 2:00 P.M. on the afternoon of proestrus, but not at 4:00 P.M., prevented this ovulation (8 out of 8 control animals ovulated, 1 out of 9 pentobarbital injected animals ovulated).

Experiment I. On a given day, rats from each cycle stage were killed by decapitation between 10:00 and 10:30 A.M. and 3:30 and 4:30 P.M. Since Everett (12) has shown that 40% of proestrous rats have already released LH by 2:45 P.M. and 90% have released LH by 3:45 P.M., and since ACTH causes adrenal ascorbic acid depletion within 20 minutes, lasting as long as three hours (10), the afternoon autopsy time was considered adequate to detect any acute ACTH release which might occur. The left adrenal was weighed and the ascorbic acid content measured by a previously described modification (8) of the Roe and Kuether method (13).

Experiment II. The previous experiment was repeated, except that some of the rats in each cycle stage received saline (1 cc./100 gm.) or pentobarbital (3 mg./100 gm.) intraperitoneally at 2:00 P.M. before the afternoon autopsy.

RESULTS

In both experiments, results obtained on animals in metestrus and diestrus were indistinguishable, and are reported as "diestrous" values. The results of Experiment 1 are summarized in Table 1. There were no significant differences in ascorbic acid concentrations between the morning

TABLE 1. ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATIONS IN PROESTROUS, DIESTROUS AND ESTROUS RATS

Cycle stage	N	Adrenal wt., mg. / Body wt., 100 gm.	Ascorbic acid, μg. / Adrenal wt., mg.
1) 10:00 to 10:30 A.M. Autopsy			
Proestrus	34	13.8 ± 1.5	4.38 ± .41
Estrus	19	14.8 ± 2.0	4.27 ± .57
Diestrus	22	14.3 ± 1.9	4.58 ± .40
2) 3:30 to 4:30 P.M. Autopsy			
Proestrus	41	14.5 ± 1.8	4.29 ± .55
Estrus	20	15.0 ± 1.9	4.37 ± .35
Diestrus	27	14.2 ± 1.3	4.61 ± .41

Values are mean ± standard deviation.

nd afternoon autopsy groups in any of the stages of the estrous cycle. The ata in Table 1 suggest that adrenal weights at estrus are higher than at iestrus. This was confirmed statistically by calculating the regression of drenal weight on body weight for estrous and for diestrous rats. An nalysis of covariance revealed a significantly ($P < .01$) higher average drenal weight in estrous rats. That the estrous hypertrophy may begin on e afternoon of proestrus is suggested by the small increase in adrenal eights in proestrous animals autopsied in the afternoon (Table 1).

The results of Experiment II are summarized in Table 2. Pentobarbital id not significantly deplete adrenal ascorbic acid in any of the cycle stages. However, five of the eight afternoon proestrous rats showed partial depletion (3.23 to 3.74 μg./mg. of ascorbic acid).

TABLE 2. EFFECT OF PENTOBARBITAL AT 2:00 P.M. ON ADRENAL WEIGHT AND ASCORBIC ACID CONCENTRATIONS IN PROESTROUS, DIESTROUS AND ESTROUS RATS

Cycle stage	N	Adrenal wt., mg. / Body wt., 100 gm.	Ascorbic acid, μg. / Adrenal wt., mg.
1) 10:00 to 10:30 A.M. Autopsy			
Proestrus	5	13.4 ± 1.0	5.08 ± .47
Estrus	3	16.3 ± 1.5	4.70 ± .33
Diestrus	3	14.3 ± 1.9	5.26 ± .26
2) 3:30 to 4:30 P.M. Autopsy: Saline or No Treatment			
Proestrus	12	15.3 ± 2.1	4.62 ± .71
Estrus	5	16.0 ± 2.1	5.01 ± .40
Diestrus	8	14.1 ± 2.4	4.99 ± .62
3) 3:30 to 4:30 P.M. Autopsy: Pentobarbital			
Proestrus	8	14.6 ± 1.0	4.11 ± .93
Estrus	5	14.3 ± 1.4	4.85 ± .40
Diestrus	7	14.9 ± 2.9	4.77 ± .95

Values are mean ± standard deviation.

DISCUSSION

The failure to find ascorbic acid depletion following LH release in the proestrous rat (Table 1) demonstrates that ACTH is not released simultaneously with the ovulating hormone. The decrease in TSH and ACTH content of rabbit pituitary glands following coitus (2) may have indicated decreased production of TSH and ACTH, rather than increased secretion, since Brown-Grant (5) failed to demonstrate changes in release of thyroidal I^{131} following coitus. It is necessary to conclude, therefore, that the pituitary can secrete gonadotrophin acutely without simultaneously secreting TSH and ACTH.

The adrenal hypertrophy at estrus is probably the result of estrogen-induced ACTH secretion (1). It has been shown that single injections of estrogen caused adrenocortical hypertrophy in 24 hours (14) and vaginal

cornification in 40 hours (15). Thus, rising titers of estrogen on the days of diestrus and proestrus are probably responsible for both these effects. However, estrogen-induced ACTH release, unlike estrogen-induced LH release (16), does not operate acutely via the 2:00 P.M. diurnal mechanism (Table 1).

Experiment II was a direct test of the suggestion by Dordoni and Timiras (11) that pentobarbital prevents LH secretion by eliciting ACTH release by virtue of a non-specific stressor effect. Since the pentobarbital did not cause a uniform stress response in animals of all cycle stages (Table 2), it seems necessary to reject this hypothesis of a *non-specific* effect of pentobarbital. Moreover, Sawyer and coworkers (17) point out that morphine and pentobarbital, both of which block gonadotrophin release, may also *block* the stress response. However, the partial ascorbic acid depletion shown by some of the pentobarbital injected proestrous rats (Table 2) may indicate that at 2:00 P.M. these animals are more susceptible to stimulation of ACTH release than are rats in other cycle stages.

Acknowledgments

Thanks are extended to Dr. John P. Marbarger of the Physical Environment Laboratory for providing environmentally controlled animal housing; and to the following students of the University of Illinois who assisted in gathering the data: Ryo Kubota, James Labes, Patrick McGuigan and John Mills.

REFERENCES

1. ZUCKERMAN, S.: In "The Suprarenal Cortex," Ed., J. M. Yoffey, Academic Press, p. 69. 1953.
2. SAXTON, J. A. AND H. S. N. GREENE: *Endocrinology* **30:** 395. 1942.
3. BOURNE, G. AND S. ZUCKERMAN: *J. Endocrinology* **2:** 283. 1941.
4. SOLIMAN, F. A. AND E. P. REINEKE: *Am. J. Physiol.* **178:** 89. 1954.
5. BROWN-GRANT, K.: *J. Physiol.* **131:** 70. 1956.
6. SELYE, H.: *Endocrinology* **25:** 615. 1939.
7. BROWN-GRANT, K., G. W. HARRIS AND S. REICHLIN: *J. Physiol.* **126:** 29. 1954.
8. SCHWARTZ, N. B., L. E. SHIRKY AND I. E. STECK: *Endocrinology* **57:** 114. 1955.
9. EVERETT, J. W.: *Ciba Foundation Colloquia on Endocrinology* **4:** 167. 1952.
10. SAYERS, G., M. A. SAYERS, T. Y. LIANG AND C. N. H. LONG: *Endocrinology* **38:** 1. 1946.
11. DORDONI, F. AND P. S. TIMIRAS: *J. Pharmacol. and Exper. Therap.* **106:** 381. 1952.
12. EVERETT, J. W.: *Endocrinology* **59:** 580. 1956.
13. ROE, J. H. AND C. A. KUETHER: *J. Biol. Chem.* **147:** 399. 1943.
14. ANDERSEN, D. H.: *J. Physiol.* **83:** 15. 1934.
15. ALLEN, E., B. F. FRANCIS, L. L. ROBERTSON, C. E. COLGATE, C. G. JOHNSTON, E. A. DOISY, W. B. KOUNTZ AND H. V. GIBSON: *Am. J. Anat.* **34:** 133. 1924.
16. SAWYER, C. H., J. W. EVERETT AND J. E. MARKEE: *Endocrinology* **44:** 218. 1949.
17. BARRACLOUGH, C. A. AND C. H. SAWYER: *Endocrinology* **57:** 329. 1955.

THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON TESTIS SIZE AND FUNCTION IN THE COCKEREL[1]

NORMAN F. BOAS

Research Division, Department of Laboratories, Norwalk Hospital, Norwalk, Connecticut

ABSTRACT

Desoxycorticosterone acetate (DCA) administered to baby chicks produced striking enlargement of the testes. Histologically this was shown to be due primarily to distention of the seminiferous tubules by intraluminal fluid.

In terms of comb weight, DCA was weakly androgenic in small doses, and in larger doses it inhibited comb growth. The latter effect probably resulted from interference with endogenous androgen production.

DESOXYCORTICOSTERONE acetate (DCA) induces striking pathological changes in various species of animals (1). Several years ago, when a study was made of the effect of steroids on the chick comb (2, 3), it was noted that chicks receiving DCA had very large testes, a finding that Selye (4) mentioned in his studies on experimental nephrosclerosis. Since no data have been presented on this unusual observation, this problem has been reinvestigated.

METHODS

Single-combed White Leghorn cockerels were received from a local hatchery one day after hatching. All experiments were started on the following day. The chicks were kept in separate experimental groups in battery brooders at a temperature of 70–80° F, and given water and chick starter mash (Libner's Blue Ribbon) *ad libitum*.

DCA[2] was dissolved in sesame oil in varying concentrations. In Experiments IV–VI, because of the high concentrations, some of the DCA remained in suspension. In all cases the chicks were given dorsal subcutaneous injections of 0.2 ml. of DCA in sesame oil or in the case of the controls, 0.2 ml. of sesame oil.

At the end of varying lengths of time (9–13 days) the animals were sacrificed with ether. They were weighed; the combs were excised according to a standard procedure (5). The testes and combs were weighed on a Roller-Smith torsion balance. The testes were reported as combined weights of both testes. All testes were placed in formalin for histological study. Representative samples were embedded, sectioned and stained with hematoxylin and eosin.

Received February 12, 1958.

[1] Supported by grants from the Norwalk Chapter, Connecticut Heart Association, and the National Institute of Arthritis and Metabolic Diseases (USPHS-A-975).

[2] Desoxycorticosterone generously supplied by Dr. Robert Gaunt, Ciba Pharmaceutical Products, Inc.

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ACETATE ON BODY AND COMB WEIGHTS OF WHITE LEGHORN COCKERELS

Expt.	Group	Days	No.	Body wt. (gm.)	Comb wt. (mg.)
I	Control	9	11	82 ± 2*	30 ± 3
	DCA (¼ mg./day)		7	82 ± 4	36 ± 5
II	Control	9	15	88 ± 1	67 ± 5
	DCA (½ mg./day)		15	88 ± 3	101 ± 6
III	Control	13	13	126 ± 4	259 ± 30
	DCA (½ mg./day)		17	133 ± 3	350 ± 24
V	Control	11	14	109 ± 2	109 ± 6
	DCA (1 mg./day)		11	116 ± 2	51 ± 3
VI	Control	10	11	92 ± 1	68 ± 6
	DCA (2 mg./day)		12	81 ± 1	45 ± 3

* S.E. Mean.

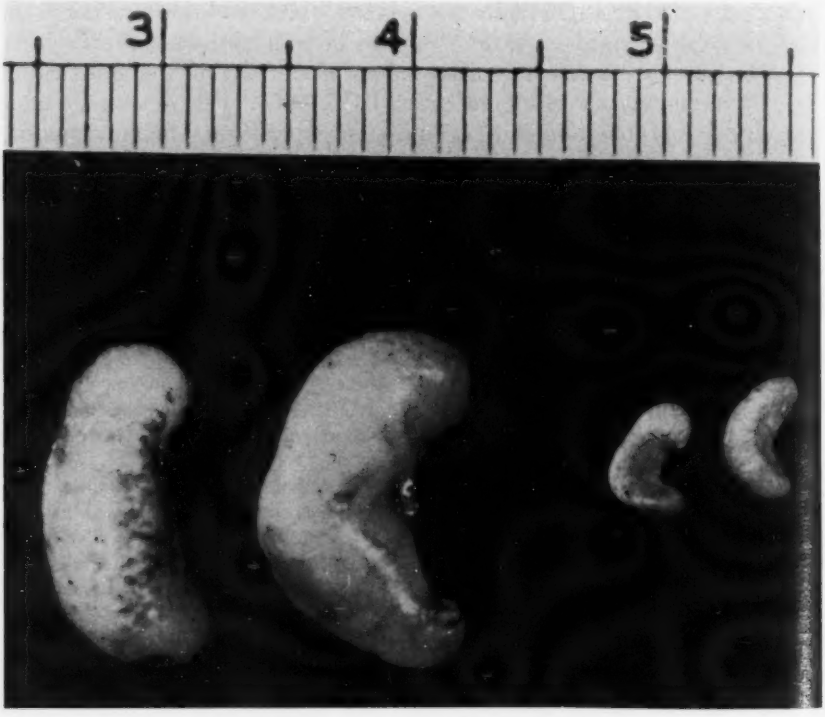


FIG. 1. Comparison of testes from DCA-treated chick (left) with normal control (right). Compare with centimeter rule.

RESULTS

DCA proved to be relatively non-toxic to the chicks. They appeared o eat well, remaining active, and with the exception of Expt. VI, the body veights were not significantly different from those of the controls. In

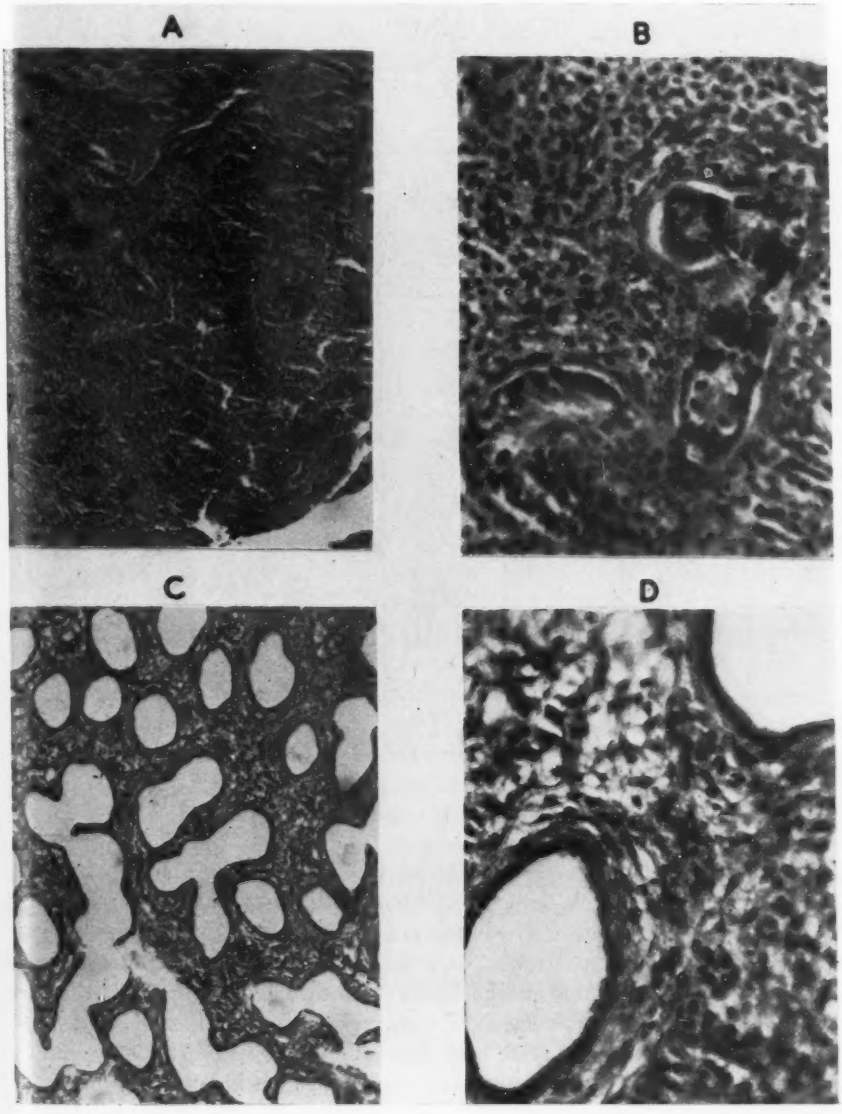


FIG. 2. Histological sections of testes from, A. 11-day-old control chick, ×60; B. same, ×240; C. chick treated 11 days with DCA, ×60; D. same, ×240.

Expt. VI, in which the animals received 2 mg./day, there was some reduction in body weight (Table 1). Many of the animals receiving DCA did demonstrate a moderate gelatinous subcutaneous edema, which may have contributed to the body weights.

Testes: The most striking feature of this group of experiments was the appearance of the testes. Testes from DCA-treated chicks were typically large, shiny and firm. On nicking the capsule, serous fluid would often escape rapidly. Figure 1 shows the relative sizes of a pair of testes from a DCA-treated chick and a control.

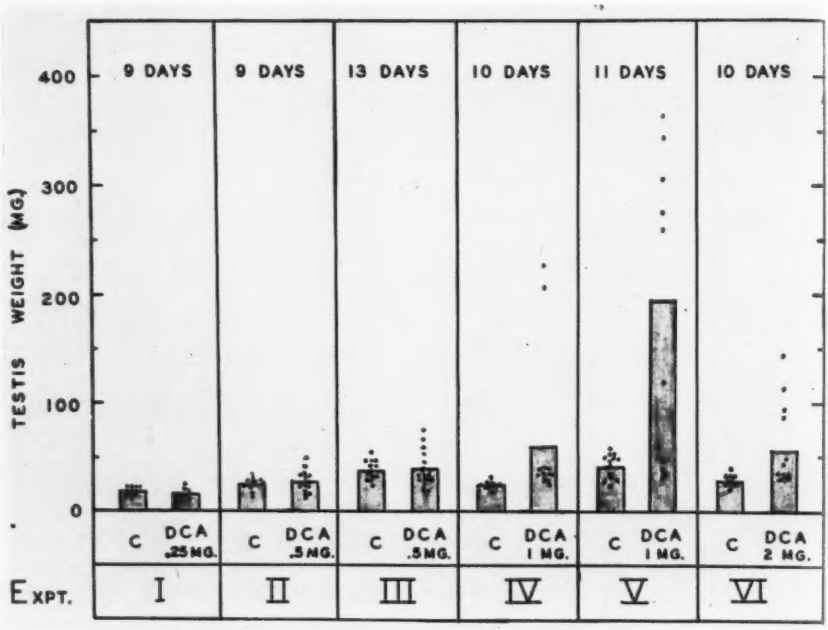


FIG. 3. Composite data on the effects of DCA on testis weights in the baby chick.

The histological appearance of these testes is shown in Figure 2. The normal 10-day-old chick testis (Fig. 2A and 2B) is a compact, relatively undifferentiated structure. The lumina of the seminiferous tubules are very small and barely demonstrable, and they contain undeveloped germinal cells. The interstital tissue compact and cellular. The testis from the DCA-treated chick, on the other hand, is strikingly different (Fig. 2C and 2D). The seminiferous tubular system is extensively dilated, with the germinal epithelium appearing to be stretched and flattened. Edema is present in the interstitial spaces as evidenced by a separation of the cellular elements and vacuolated spaces in the interstitium.

Figure 3 summarizes all the testis weight data obtained from six experiments in which DCA was administered to 2-day-old cockerels for periods varying from 9–13 days. The larger doses of DCA had a greater effect on testis weight than did the smaller doses. In each experiment there were one or more pair of testes larger than any of the controls. In all cases there was considerable overlap in data between the experimental groups and their controls. Nevertheless, in Expts. IV–VI, some of the testes were so large as to leave no doubt of the significance of this response. Because of the apparent unpredictability of the response to DCA and the unresponsiveness of some testes in each group, it was not possible to work out a good dose-response curve.

Combs: Combs were weighed primarily to ascertain whether the swelling properties of DCA on the testes influenced the rate of androgen production by the testes. The data are shown in Table 1. There were no significant differences in comb weights in Expt. I in which the treated group received 0.25 mg. DCA/day. The chicks in Expts. II and III, receiving $\frac{1}{2}$ mg. DCA/day, had significant increases in their comb size. Animals receiving 1–2 mg. DCA/day had significantly smaller comb weights.

DISCUSSION

The profound effect of DCA on the chick testis cannot easily be explained. The edema of the interstitium is perhaps no greater than that seen in other tissues. The distention of the seminiferous tubules, on the other hand, appears to be a specific organ effect of DCA, not seen with other potent salt-retaining steroids (9-α-fluoro F, 2-methyl-9α fluoro F, and Δ'-9α fluoro F) (6). This finding has not been reported in other species. In theory it could be produced by an imbalance in formation of tubular fluid (over-secretion; decreased reabsorbtion) or obstruction of a vas deferens. This question cannot be answered as yet.

The androgenic properties of DCA are very weak. Some have reported comb stimulation in the chick and capon (7–9); others have observed no effect (10, 11). The data here suggest that DCA may be androgenic in small doses, but it actually inhibits comb growth in larger doses. This could be explained either on the basis of interference with normal Leydig cell function or an inhibition of pituitary gonadotrophin production as is seen with estrogens (3).

Acknowledgments

We wish to thank Dr. Roy Barnett for taking the photomicrographs and Miss Dorothy Skoglund for her technical assistance.

REFERENCES

1. Selye, H. and C. E. Hall: *Arch. Path.* **36:** 19. 1943.
2. Ludwig, A. W. and N. F. Boas: *Endocrinology* **46:** 291. 1950.

3. Boas, N. F. and A. W. Ludwig: *Endocrinology* **46:** 299. 1950.
4. Selye, H.: *Can. Med. Assn. J.* **47:** 515. 1942.
5. Frank, R. T., E. Klempner, F. Hollander and B. Kriss: *Endocrinology* **31:** 63. 1942.
6. Boas, N. F.: To be published.
7. Benoit, J., G. Gros, R. Paris and R. Kehl: *Compt. Rend. Soc. Biol.* **136:** 67[illegible]. 1942.
8. Morato Manero, J.: *Arch. Clin. Inst. Endocrinologie* **1:** 343. 1937–40.
9. Mussio Fournier, J. C. and A. S. Albrieux: *Arch. Clin. Inst. Endocrinologie* **1:** 333. 1937–40.
10. Paschkis, K. E.: *Proc. Soc. Exp. Biol. Med.* **46:** 336. 1941.
11. Caridroit, F.: *Compt. Rend. Soc. Biol.* **138:** 147. 1944.

SENSITIZATION TO HYPERTENSIVE ACTION OF DESOXYCORTICOSTERONE BY UNILATERAL NEPHRECTOMY: RELATIONSHIP TO DOSAGE AND TO INTERVAL BETWEEN SURGERY AND HORMONE ADMINISTRATION[1]

C. E. HALL AND O. HALL

The Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas

ABSTRACT

The hypertensive potency of desoxycorticosterone (DCA), at a dosage of 1 mg./day, was assessed in animals subjected to uninephrectomy. Those in which the operation was performed on the day of beginning hormone treatment were compared with others operated, respectively, 7 and 14 days earlier. Although the response was essentially the same in the latter two groups, hypertension and its consequences were earlier in onset and more severe in degree in both than in the first. A second experiment compared newly nephrectomized animals with those operated 21 days earlier, and in which compensatory renal hypertrophy was therefore complete, at dosages of 2 mg./day and 0.5 mg./day. No difference in response was detectable. It is concluded that there is a relative refractoriness to the hypertensive effects of the steroid during the early and most rapid phase of renal compensatory hypertrophy. This difference is best revealed by dosages in the range of 1 mg./day. Double this dosage seems to overwhelm such differences in sensitivity, whereas at half of it the steroid fails to be effective in the period when a real difference exists in the degree of renal hypertrophy and therefore in sensitivity.

A RECENT publication from this laboratory presented evidence indicating that although unilateral nephrectomy sensitizes the rat to the hypertensive cardiovascular disease induced by desoxycorticosterone acetate (DCA) and sodium loading, animals in which hormone treatment is instituted on the day of kidney removal are much less responsive than those in which two weeks is allowed to elapse between the operation and beginning hormone administration (1, 2). This was indicated by earlier elevation of blood pressure, involvement of a larger number of animals and greater incidence and severity of the cardiovascular lesions in the latter as compared to the former. It was interpreted as possibly indicative of a relative resistance to the sodium retaining effect of DCA-treatment during the active phase of renal compensatory hypertrophy, and implies that by whatever mechanism unilateral nephrectomy sensitizes animals to DCA-treatment, it is not simply a matter of reduction in renal mass.

Received March 15, 1958.

[1] This study was supported by a research grant (RG 4833) from the National Heart Institute of the United States Public Health Service.

It was the object of the first of the experiments reported herein to evaluate the importance of the time interval interposed between the operation and steroid treatment to the difference in sensitivity; and of the second experiment to ascertain whether the difference could be accentuated further by varying the dosage of hormone, in this case by doubling and halving the dosage which earlier experiments had shown to be effective, inasmuch as there was no reason to believe that the most appropriate dosage had initially been selected.

MATERIALS AND METHODS

Female rats of the Holtzman strain were used throughout, and Purina Laboratory Chow was fed *ad lib.* In the first experiment 40 animals 55–75 gm. in weight were divided into four groups having the same average body weight. The left kidney was removed, under ether anesthesia, from the sixteen animals of Group 1; a week later the same was done to the animals of Group 2, and in a further week to those of Groups 3 and 4, each composed of eight animals.

From this time until the experiment was terminated all animals were placed on 0.85% NaCl drinking solution, and a water suspension of DCA[2] was administered to animals of Groups 1, 2 and 3 at a dosage of 1 mg./day in 0.2 cc. of fluid, and the suspending medium in this same volume to those of Group 4. Blood pressure was measured each week by a tail plethysmograph method (3) on unanesthetized animals. The average of 3 consecutive readings agreeing to within 5 mm. of Hg was taken as representative of the blood pressure of each animal and values in excess of 150 mm. Hg were regarded as hypertensive. The experiment was terminated on the 28th day. At autopsy the viscera were examined for cardiovascular lesions and the cardiac ventricles and kidneys dissected free of adherent connective tissue, weighed to the nearest mg. on an accurate torsion balance and fixed for histologic examination.

In Experiment 2, fifty female rats 55–75 gm. were divided into 5 equal groups. The left kidney was then removed from the animals of Groups 2 and 4, and three weeks later the same was done to animals in Groups 1, 3, and 5.

At this time all groups were placed on 0.85% NaCl drinking solution, and from then until the experiment was terminated the animals in Groups 1 and 2 received 2 mg./day DCA in 0.2 cc. of suspending medium; those in Groups 3 and 4 received 0.5 mg./day in the same volume and those of Group 5 the medium only. Blood pressures were measured and assessed as in the previous experiment. The experiment was terminated after 28 days of hormone administration, the animals killed with ether, and the hearts, kidneys and samples from the mesenteric arteries were fixed for subsequent histologic examination.

RESULTS

In the first experiment, hypertension was earlier in onset, of greater magnitude and affected a larger percentage of the animals in Groups 1 and 2 than in Group 3. By the second week of hormone administration two thirds of the animals subjected to left nephrectomy two weeks prior to hormone treatment and three quarters of those in which the operation preceded treatment by one week were hypertensive, whereas none of those in which hormone treatment was begun on the day of kidney removal had

[2] The hormone was generously supplied by Dr. Robert Gaunt of the Ciba Pharmaceutical Co. Summit, N. J.

yet developed an elevation of arterial pressure in excess of 150 mm. Hg. From then on, the number of hypertensive animals in each group, and the average blood pressure of the group began to increase steadily; and by the fourth week of treatment, although it was still evident that the animals in Groups 1 and 2 were more severely affected than those in Group 3, it was also equally clear that the gap was being rapidly closed and soon would be eradicated. Renal and cardiac arteriopathy and associated lesions, so thoroughly described elsewhere (4–6) as to obviate extensive treatment here, although encountered with equal frequency in Groups 1 and 2, was rarely seen in Group 3 and not at all in Group 4. The cardiac ventricles of both Group 1 and Group 2 animals, expressed as mg. per 100 gm. body weight differed significantly in weight ($P < 0.001$) from controls; whereas those of Group 3 animals did not ($P > .05$). The data are summarized in Figure 1 and Table 1.

In the second experiment the blood pressures were clearly related to the quantity of hormone, but at the dosages employed there was no apparent relationship between the time which had been allowed to elapse between nephrectomy and hormone administration, and the pressor response. At the higher dose all except one animal in each of the treated groups were hypertensive by the end of the first week of treatment; whereas at the lower dosage even by the fourth week between 25% and 50% of the animals in both groups still had not developed blood pressures in excess of 150 mm. Hg. At autopsy the cardiovascular lesions which constitute a prominent

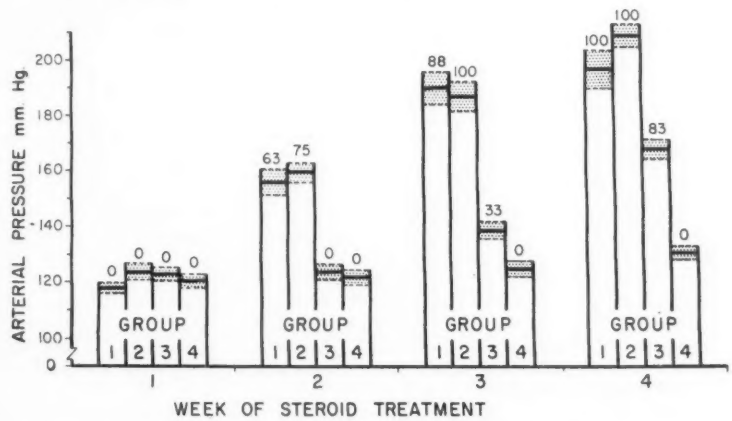


FIG. 1. Blood pressure averages at weekly intervals. The solid line at the top of the columns indicates the group average, the shaded area above and beneath encompasses the standard error of the measurement, and the superscript figure indicates the percentage of each group having arterial pressures in excess of 150 mm. Hg. Groups 1, 2 and 3 each received 1 mg./day DCA and were subjected to unilateral nephrectomy respectively 14 days, 7 days and 0 days before hormone treatment began. Group 4 are controls and were uninephrectomized at the same time as Group 3 animals.

TABLE 1. TREATMENT DATA, ORGAN WEIGHTS AND PATHOLOGIC CHANGES IN DCA-TREATED RATS

Day	Data	Group			
		1	2	3	4
	Left Nephrectomy on day	0	7	14	14
0	No. animals	16*	8	8	8
	Body wt., gm.	68 ± 3†	68 ± 3	68 ± 3	76 ± 3
14	0.85% NaCl to drink	+	+	+	+
	1 mg./day DCA	+	+	+	−
	Body wt., gm.	142 ± 5	139 ± 5	129 ± 5	136 ± 7
42	No. animals	8	8	6	8
	Body wt., gm.	175 ± 6	161 ± 8	174 ± 19	188 ± 7
	Heart wt., mg.	789 ± 37	752 ± 32	682 ± 26	679 ± 16
	mg.%	451 ± 21	469 ± 13	408 ± 31	351 ± 1
	Kidney wt., mg.	2111 ± 92	1796 ± 65	1885 ± 42	1633 ± 41
	mg.%	1204 ± 51	1122 ± 45	1131 ± 59	880 ± 30
	Autopsy findings				
	Renal lesions				
	% incidence	63	50	0	0
	avg. severity	+	+	0	0
	Cardiac lesions				
	% incidence	63	75	17	0
	avg. severity	+	+	< +	0

* 8 killed on 14th day.
† ±S.E. of Mean.

TABLE 2. EFFECT OF HORMONE DOSAGE ON CARDIOVASCULAR RESPONSES TO DCA

Day	Data	Group				
		1	2	3	4	5
0	Dose of DCA in mg./day	2	2	0.5	0.5	0
	No. animals	10	10	10	10	10
	Body wt., gm.	145 ± 5*	146 ± 5	140 ± 3	140 ± 7	150 ± 7
	Blood Press., mm. Hg	123 ± 3	128 ± 4	126 ± 3	130 ± 3	124 ± 2
7	Blood Press., mm. Hg	161 ± 3	160 ± 5	133 ± 5	137 ± 4	127 ± 3
14	Blood Press., mm. Hg	187 ± 11	187 ± 13	139 ± 7	138 ± 6	132 ± 3
21	Blood Press., mm. Hg	190 ± 7	196 ± 11	153 ± 8	153 ± 9	135 ± 4
28	No. animals	9	8	9	8	7
	Body wt., gm.	184 ± 5	180 ± 6	193 ± 6	183 ± 10	198 ± 7
	Blood Press., mm. Hg	197 ± 8	199 ± 10	163 ± 10	173 ± 11	134 ± 4
	% group hypertensive	100	100	55	75	0
	Autopsy findings:					
	Renal lesions					
	% incidence	78	88	0	13	0
	avg. severity	+ +	+ +	0	< +	0
	Cardiac lesions					
	% incidence	89	88	0	13	0
	avg. severity	+ +	+ +	0	< +	0

* ±S.E. of Mean.
Odd numbered groups uninephrectomized on day 0, even numbered groups 21 days earlier.

finding in rats with severe hypertension were, with a single exception, confined to animals receiving the higher dose of hormone and equally abundant and severe in both groups at this dosage. The data are summarized in Table 2.

DISCUSSION

The evidence obtained from the first experiment was entirely confirmatory of that obtained in similar earlier experiments and indicates that uninephrectomized rats are more senstive to the hypertensive effects of suitable quantities of DCA when a degree of compensatory hypertrophy has been allowed to take place before treatment is begun, than those in which the hormone treatment is instituted on the day of operation. This experiment indicates, in addition, that the protective mechanism operative in the latter is one which is largely if not entirely confined to the first postoperative week; there being no difference in response between animals nephrectomized one week before hormone treatment and those in which a two-week interval was interposed between surgery and hormone treatment. It thus coincides with the most active phase of compensatory renal hypertrophy (7) and is probably related to some physiologic characteristics of this stage, as has been discussed elsewhere (1, 2).

The left kidneys of Group 1 animals weighed 498 ± 15 mg./100 gm. body wt. at extirpation. Two weeks later the left kidneys of Group 3 animals weighed 417 ± 13 mg./100 gm. at extirpation. Eight animals of Group 1 were killed at this time and the right kidneys were found to weigh 716 ± 24 mg./100 gm. When hormone treatment began, therefore, animals of Group 1 had kidneys which were 72% larger than those of Group 3. Since the former were much more responsive to treatment than the latter, it may safely be inferred that the sensitization afforded by uninephrectomy cannot be ascribed simply to a reduction in functional renal mass, at least insofar as this can be accurately assessed by weight. The response of animals subjected to renal constriction by a figure-of-eight ligature indicates also "that if an incretory function of the kidney regulates blood pressure it is not a function of renal mass" (8).

The failure to elicit such a difference in response at either the 2 mg./day or the 0.5 mg./day steroid levels was unexpected but understandable. At the high dosage the protective mechanism which exists during the most active phase of kidney hypertrophy seems to have been overwhelmed by the metabolic dysfunction induced by DCA, hence all animals responded, regardless of the stage of kidney growth. At the low dosage, on the other hand, the hormone was so weakly active that by the time it began to exert an effect, there had ceased to be any real difference between the groups in respect to the completeness of compensatory renal hypertrophy. As the first experiment indicated, it is the first week of active compensatory hy-

pertrophy which is all important, hence any hormonal effect which is not effective within this period is unlikely to be determinant in the differences in sensitivity which differentiates newly uninephrectomized animals from those of longer standing. Furthermore compensatory renal hypertrophy is complete or virtually complete in the rat at three weeks (7). Therefore after three weeks of hormonal treatment, the renal status of animals uninephrectomized at the beginning of hormone treatment will, in respect to compensatory hypertrophy, be the same as that of littermates operated at any time earlier, and no difference in sensitivity would be expected. The elicitation of differences in pressor effects of DCA as related to completeness of renal hypertrophy in the rat thus seems to be best demonstrated with doses close to 1 mg./day.

Previous experiments, and the first of those reported herein, compared the response of animals in which uninephrectomy was either coincident with beginning hormone treatment or preceded it by either one or two weeks, hence all kidneys were undergoing compensatory hypertrophy during at least a part of the treatment period, but at different rates. In the second of the present experiments the comparison was between newly uninephrectomized animals and those operated three weeks earlier. Inasmuch as compensatory hypertrophy is complete in this period of time, the comparison was then between the response of animals with rapidly hypertrophying kidneys and those in which such growth had virtually, if not entirely, ceased. This alone may have accounted for the failure to demonstate a difference in sensitivity in the second experiment. Although this seems to be a less attractive explanation of the findings than that presented earlier, the data given herein do not permit of a definitive distinction between the alternatives.

REFERENCES

1. Hall, C. E. and O. Hall: *Fed. Proc.* (in press).
2. Hall, C. E. and O. Hall: *Am. J. Physiol.* (in press).
3. Sobin, S. S.: *Am. J. Physiol.* **146:** 179. 1946.
4. Selye, H. and E. I. Pentz: *Canad. Med. Assoc. J.* **49:** 264. 1943.
5. Masson, G. M. C., J. B. Hazard and A. C. Corcoran: *Arch. Path.* **49:** 641. 1950.
6. Selye, H., C. E. Hall and E. M. Rowley. *Canad. M. Assoc. J.* **49:** 88. 1943.
7. Rollason, H. D.: *Anat. Rec.* **104:** 263. 1946.
8. Shapiro, A. P. and J. Melhado: *Proc. Soc. Exper. Biol. and Med.* **96:** 619. 1957.

STUDIES ON SQUALENE POTENTIATION OF THYMIC RESPONSE TO CORTICOTROPIN[1,2]

IRENE T. KLINE

Division of Endocrinology, Henry Ford Hospital and the Department of Biochemistry, Edsel B. Ford Institute for Medical Research, Detroit, Michigan
and the
Departments of Obstetrics and Gynecology, and Biochemistry, University Hospitals and Western Reserve University School of Medicine, Cleveland, Ohio

ABSTRACT

It has been suggested on the basis of previously presented data that exogenous squalene potentiates response of the thymus to corticotropin (ACTH) in hypophysectomized rats by serving as a readily utilized precursor of glucocorticoids. Other possible explanations have now been studied further. Attempts to demonstrate that the potentiation occurs because squalene acts as a stress-producing agent or directly as a thymolytic agent have given negative results, even in animals with impaired adrenal-pituitary homeostatic pathways, sensitized or conditioned with glucocorticoids.

Potentiation has been erratic in occurrence and in degree, but it has now become possible to rationalize these inconsistent results. The data indicate that potentiation can be detected by thymus involution in hypophysectomized rats when the levels of ACTH employed permit the increments of corticoids attributable to a given dose of squalene to represent a measurable proportion of the total glucocorticoids synthesized. When this situation obtains potentiation occurs even in an experimental period as short as twenty-four hours.

SQUALENE injected subcutaneously for seven days enhances the effectiveness of adrenocorticotropin (ACTH) as measured by adrenal weight increase and thymic involution in hypophysectomized male rats (1). This evidence is consistent with the idea that exogenous squalene can serve as an easily utilized precursor of glucocorticoids *in vivo* and supports the hypothesis that squalene is a natural intermediate in steroid biogenesis (2, 3).

Acute involution of the thymus is a measure of glucocorticoid action (4, 5) but only under conditions which ensure the absence of other thymolytic factors (6). Attempts to identify squalene as a thymolytic agent have given negative results. No thymolytic action of squalene *per se*, indirect or direct, was observed in intact, adrenalectomized or hypophysectomized rats under carefully controlled experimental conditions (1). Not ruled out

Received April 2, 1958.

[1] This work was supported in part by Research Grants A-309 and A-1092, National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

[2] Preliminary reports of some of this work were presented at the Laurentian Hormone Conference, 1955 and at the Federation Meetings, 1958.

by previous experimental work (1), however, was the possibility that a direct thymolytic effect of squalene, an effect not mediated through the adrenal, might be revealed in the absence of hormonal homeostatic mechanisms if the thymic tissue were "conditioned" by corticoids (7). Data herein presented are an indication that glucocorticoids do not have a role in permitting a direct effect of squalene on the thymus to become apparent, since squalene has not been found to enhance the thymolytic action of corticosteroids in either hypophysectomized or adrenalectomized rats.

Studies are also reported which show that potentiation by squalene of adrenal and thymic responses to ACTH can occur in a much shorter experimental period than was first used. Results obtained with a two-week experimental period have been confirmed in experiments of only one week's duration, and augmentation has been observed within twenty-four hours after the beginning of ACTH treatment.

PROCEDURES

All rats were of the Sprague-Dawley strain.[3] They were shipped when twenty-two days of age and received in the laboratory the next day. Hypophysectomized rats were operated at 21 days of age, one day before shipment, but those which were adrenalectomized and castrated were operated the day after arrival in the laboratory, at twenty-four days of age. All rats were fed *ad libitum* a diet of canned meat (Pard), milk, whole wheat bread soaked in water, lettuce, carrots and oranges. Adrenalectomized rats received 0.9% NaCl instead of tap water.

Three types of experiments differing primarily in duration of ACTH treatment periods were done. ACTH was given for 5 days in the two-week experiment (Table 1), for 3 days in the one-week experiments (Fig. 1 and Table 3) and for 1 day in the five-day experiment (Table 3). Experiment 61M, a two-week study, was identical in plan to experiments reported previously (1) except that groups given cortisol acetate, and cortisol acetate plus squalene were included. Squalene was administered daily for seven days from 27 through 33 days of age. ACTH and cortisol acetate were each given during the last 5 of these 7 days. This schedule allowed 8 days for adrenal regression following hypophysectomy and prior to the beginning of treatment with ACTH. In the one-week experiments (66M, 71M and 75M), ACTH, cortisol acetate, squalene and mineral oil were all given for 3 days, after allowing 3 days for postoperative adrenal regression in hypophysectomized animals and 2 days for surgery and recovery therefrom in adrenalectomized-castrated animals before the beginning of treatment. In Experiment 19D, squalene was administered the afternoon of the twenty-fourth day of age and both squalene and ACTH were injected at 9 A.M. and 5 P.M. on the twenty-fifth day.

Autopsy was performed in all experiments 16 to 24 hours after the last injections, except that groups indicated as initial controls were sacrificed the day injections were begun in the experimental groups. At autopsy, after an overdose of chloroform, thymi and adrenals were quickly excised, trimmed, and weighed to the nearest 0.2 mg. on a Roller-Smith torsion balance.

All substances were injected subcutaneously but at sites far enough removed from each other to preclude mixing under the skin when more than one substance was administered. ACTH[4] was given as a single injection of 2 Armour Units per 0.1 ml. gel

[3] From Hormone Assay Laboratories, Inc., Chicago, Illinois.

[4] Armour's ACTHar Gel, 20 Armour Units or 20 I.U. per ml. Dilution for Exp. 19D was with the gel medium, supplied through the courtesy of Dr. E. A. Fullgrabe, of the Armour Laboratories.

once a day in Experiment 66M and 75M, and of 1.5 Armour Units per 0.075 ml. gel in Experiment 61M. In Experiment 19D, each of the two injections of ACTH was 0.5 I.U. in 0.1 ml. gel. Each dose of squalene[5] or mineral oil[6] was 0.2 ml., injected as 0.05 ml. at each of four closely adjacent sites, in all experiments. Cortisol acetate[7] was administered in the amount of 50 μg. per 0.1 ml. of a saline suspension once per day in Experiment 61M, and twice per day in the amount of 30 μg./0.1 ml. of a saline suspension in Experiment 71M and 75M.

RESULTS

Two-week experimental period (Exp. 61M, Tables 1 and 2)

From the tabulated data it is apparent that squalene does not change the thymic-involuting action of cortisol acetate under experimental conditions parallel to those which permitted augmentation of response to ACTH

TABLE 1. (EXPERIMENT 61M) AUGMENTATION BY SQUALENE OF RESPONSE TO ACTH BUT NOT TO CORTISOL ACETATE IN HYPOPHYSECTOMIZED RATS TREATED FIVE DAYS

Treatment (details in text)	No. of rats	Autopsy data: Mean values ± S.E.		
		Body weight, gm.	Thymus, mg./100 gm. body weight	Adrenals, mg./100 gm. body weight
None (initial controls)	8	46.5 ± 1.5	321.6 ± 23.0	22.3 ± 0.6
None (final controls)	8	59.3 ± 1.1	326.8 ± 24.7	13.7 ± 0.6
Squalene	7	63.1 ± 2.4	275.3 ± 17.5	15.1 ± 0.7
ACTN	8	58.4 ± 1.7	261.9 ± 12.9	20.2 ± 1.2
ACTH + squalene	7	54.7 ± 1.6	125.0 ± 14.5	25.9 ± 1.3
Cortisol acetate	8	56.4 ± 1.9	255.8 ± 21.2	15.0 ± 0.9
Cortisol acetate + squalene	8	59.0 ± 2.4	264.3 ± 12.8	18.1 ± 1.7

TABLE 2. STATISTICAL ANALYSIS OF DATA IN TABLE 1; P VALUES

Treatments compared	Basis of comparison	
	Relative thymus wt.	Relative adrenal wt.
ACTH vs. ACTH + squalene	<0.001	0.01–0.001
Cortisol acetate vs. cortisol acetate + squalene	0.5	0.2 –0.1
ACTH vs. final controls	0.05–0.02	0.001
Cortisol acetate vs. final controls	0.05	0.3 –0.2
Cortisol acetate + squalene vs. final controls	—	0.05–0.02
Squalene vs. final controls	0.2 –0.1	0.2 –0.1
ACTH + squalene vs. initial controls	<0.001	0.05–0.02

in five days. Although the amount of ACTH which by itself caused a 20% decrease in relative thymus weight was three times as effective in this regard in squalene-treated animals, cortisol acetate, at a dose level which also caused a 20% decrease in relative thymus weight was not augmented in effectiveness by simultaneous squalene treatment. Tendencies toward maintenance of adrenal weight by squalene or cortisol acetate individually were

[5] Purchased from Distillation Products Industries, Division of Eastman Kodak Co., Rochester 3, New York.

[6] U.S.P. heavy.

[7] Sharpe and Dohme's Hydrocortone Acetate, 25 mg. per ml., diluted with 0.9% NaCl.

additive when both substances were given. ACTH alone maintained relative adrenal weights at pre-injection levels but in squalene-treated animals the same dose of ACTH produced adrenals significantly larger than those of the initial controls. Neither thymus nor adrenal weights were significantly different from final control levels in animals given only squalene.

One-week experimental period (Exp. 75M, Fig. 1; Exp. 66M, 71M and 191, Tables 3 and 4)

From the graph (Fig. 1) it can be seen that in Experiment 75M the dose of ACTH used caused a small but significant decrease of 17% in relative thymus weight. This same dose of ACTH administered to animals also treated with squalene produced a 44% decrease in relative thymus weight, a response two to three times as great as that which occurred with ACTH alone. Simultaneous treatment with mineral oil, a mixture of saturated

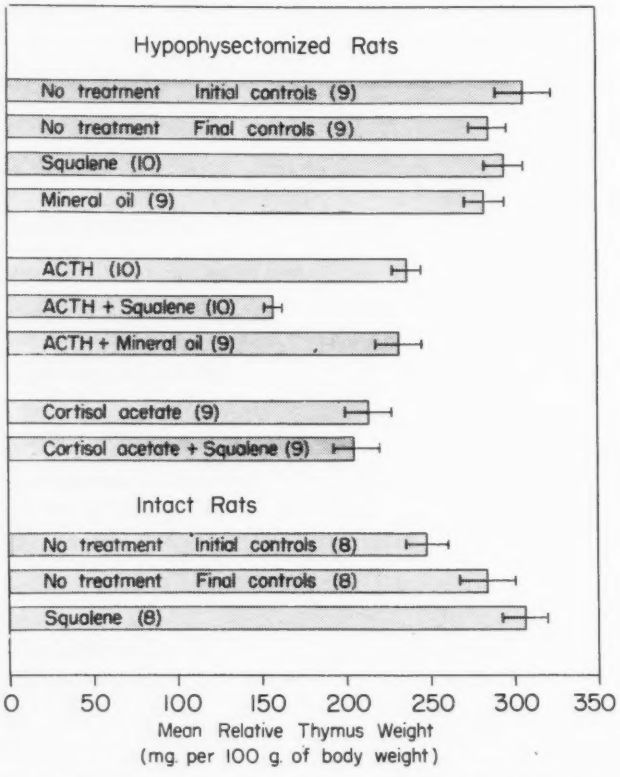


FIG. 1 (Exp. 75M). Potentiation by squalene of ACTH-induced thymic regression in hypophysectomized rats. Explanation is given in text. Numbers of animals per group are shown in parentheses.

TABLE 3. AUGMENTATION BY SQUALENE OF RESPONSES TO ACTH BUT NOT TO CORTISOL ACETATE IN RATS TREATED THREE DAYS OR ONE DAY

Experiment	ACTH treatment period (days)	Treatment (details in text)	No. of rats	Autopsy data: Mean values ± S.E.		
				Body weight, gm.	Thymus, mg./100 gm. body weight	Adrenals, mg./100 gm. body weight
66M Hypophysectomized Rats	3	None	8	54.1 ± 1.2	333.6 ± 17.4	19.9 ± 0.8
		Squalene	8	57.9 ± 1.2	315.2 ± 10.7	21.9 ± 0.7
		ACTH	8	53.6 ± 1.1	188.5 ± 14.3	30.9 ± 0.7
		ACTH + squalene	8	52.3 ± 1.9	109.7 ± 6.7	35.4 ± 1.3
71M Adrenalectomized-Castrated Rats	3	None	9	65.9 ± 2.5	421.4 ± 16.7	—
		Squalene	9	65.3 ± 2.4	418.5 ± 33.1	—
		Cortisol acetate	9	66.9 ± 1.6	310.3 ± 13.3	—
		Cortisol acetate + squalene	9	67.2 ± 2.3	276.5 ± 14.3	—
19D Hypophysectomized Rats	1	None	8	50.5 ± 1.7	350.7 ± 17.3	22.0 ± 1.7
		Squalene	8	51.0 ± 1.1	334.4 ± 15.3	22.1 ± 1.8
		ACTH	5	50.6 ± 1.2	263.9 ± 10.7	25.5 ± 1.3
		ACTH + squalene	6	53.2 ± 1.1	222.3 ± 9.6	28.5 ± 2.4

hydrocarbons, did not change the thymolytic response to ACTH. A dose of cortisol acetate which alone caused a 25% decrease in relative thymus weight was not augmented in squalene-treated animals, even though 75% of the thymus was still available for measuring any synergism which might have occurred between these two substances. No effect of squalene alone on the thymus occurred in either intact or hypophysectomized controls. Only the data for thymus weights have been graphed since there was no augmentation of adrenal response to ACTH in this experiment. ACTH did maintain the relative adrenal weight (25.5 ± 0.9 mg. before injection as compared to 27.5 ± 2.3 mg. at autopsy) but was not significantly increased in effectiveness by squalene treatment (29.0 ± 2.5 mg.).

In Experiment 66M the same dose of ACTH as used in Experiment 75M produced a 43% decrease in relative thymus weight in contrast to the 17% decrease in Experiment 75M. Even though the thymolytic response to the ACTH alone was as great as the augmented response in Experiment 75M, significant potentiation by squalene still occurred, the relative thymus weight having been reduced by 67% in the group given both substances. This represents only a 50% increase in effectiveness of the ACTH on the basis of decrease in thymus weight, instead of a 100 to 200% increase as

TABLE 4. STATISTICAL ANALYSIS OF DATA IN TABLE 3; P VALUES

Exp. No.	Treatments compared	Basis of comparison	
		Relative thymus wt.	Relative adrenal wt.
66M	ACTH vs. ACTH + squalene	<0.001	0.01–0.001
	ACTH vs. none	<0.001	<0.001
71M	Cortisol ac. vs. cortisol ac. + squalene	0.1	—
	Cortisol acetate vs. none	<0.001	—
19D	ACTH vs. ACTH + squalene	0.02–0.01	0.3 –0.2
	ACTH vs. none	0.001	0.2 –0.1
	None vs. ACTH + squalene	<0.001	0.05–0.02

found in Experiment 75M. In Experiment 66M, however, ACTH-stimulation of adrenal weight increase was significantly augmented by squalene. This has been observed consistently with 5-day treatment periods but was not found in Experiment 75M or in other experiments, not reported here, in which 3-day treatment periods were used.

In adrenalectomized-castrated male rats (Table 4, Exp. 71M), the thymolytic effect of cortisol acetate was not significantly altered by simultaneous treatment with squalene, nor did squalene *per se* have a thymolytic action.

One-day ACTH treatment (Exp. 19D, Tables 3 and 4)

ACTH alone produced a 25% decrease in relative thymus weight, while in animals also treated with squalene, ACTH caused a decrease in relative thymus weight of 37%. The 12% difference represents a statistically significant augmentation of ACTH by squalene. Parallel effects on the relative adrenal weights were not observed. It should be noted, however, that, even though ACTH alone did not maintain the adrenals at a relative weight significantly higher than that of the untreated controls, a greater relative adrenal weight was found in animals treated with both ACTH and squalene.

DISCUSSION

Experimental results have been presented which are consistent with the idea that squalene potentiates the thymolytic action of ACTH in hypophysectomized rats indirectly by serving as an easily utilized precursor of glucocorticoids rather than directly as a thymolytic agent.

Ingle (7) has advanced the concept that "the presence of cortical hormones is required to support normal metabolic responsiveness to stress." On the other hand, Selye and Horava (8), and Herlant (9) have suggested that stress increases responsivity or sensitivity to adrenal steroids. The possibilities existed therefore that, if the adrenal-pituitary pathway of homeostatic regulation were impaired, squalene might be shown to have a direct thymolytic effect, as a stressing agent, in rats "conditioned" with glucocorticoids, or that glucocorticoids might cause increased thymic regression in rats also given squalene.

Data from three experiments designed to test these possibilities do not support such explanations of the augmentation of ACTH by squalene. Squalene did not change the thymolytic response to cortisol acetate in hypophysectomized rats in either of two experiments (Table 1 and Fig. 1). In groups run simultaneously, however, squalene did increase two- to threefold the effectiveness of ACTH, the dose of ACTH having been adjusted so as to bring about the same degree of thymic regression as did the dose of cortisol acetate given. In adrenalectomized-castrated rats (Table 3), with thymus weights at a highly sensitive level, cortisol acetate in a dose which

decreased relative thymus weight by 26% caused a 34% decrease in animals also given squalene. This difference is not statistically significant ($P=0.1$) and certainly is not comparable to the observed two- to threefold increase in effectiveness of ACTH. It is therefore probable that squalene augments ACTH otherwise than by imposing stress.

Failure of squalene to enhance glucocorticoid action may therefore be interpreted as additional indirect evidence consistent with the idea that squalene augments ACTH action because it is utilized as a precursor of glucorocticoids. Squalene augmentation of response to ACTH in experiments of short duration, even with only twenty-four hours of ACTH treatment, implies, furthermore, that squalene is a preferentially utilized precursor of glucocorticoids, i.e., that it is used even prior to exhaustion of stores of other precursor substances. Such preferential utilization of squalene in liver cholesterol biosynthesis has been suggested by results of work with liver slices (11). Loud (12) has interpreted studies on the turnover of liver squalene in relation to cholesterol synthesis *in vivo* to mean that the squalene of rat liver is "separable metabolically into a small active component (possibly enzyme-bound) and a larger, relatively inert pool." If a parallel situation exists in the adrenal, exogenous squalene, in potentiating response to ACTH, appears to be utilized more as the "active component" than as part of a relatively inert pool.

If, as proposed by Haynes and Berthet from *in vitro* studies (13), one major mechanism by which ACTH stimulates corticosteroid synthesis *in vivo* is by promoting the generation of reduced triphosphopyridine nucleotide (TPNH), it may well be that supplying exogenous precursor permits a better utilization of the TPNH generated under ACTH influence.

Koritz and Péron (14) have recently reported results from studies *in vitro* which indicate that in addition to stimulating the formation of reduced TPN, ACTH also acts to make corticoid precursor available in a controlled manner, gradually but never completely, from some "enzymatically unavailable" storage form, for corticosteroidogenesis. Freezing the adrenal tissue, however, is postulated by them (14) to release the bound precursors all at once in an uncontrolled manner. They found that tissue previously frozen, although unable to respond to additional ACTH (13), could respond dramatically with increased corticosteroid production, when TPNH availability was increased (14), even without added ACTH. Even at maximal ACTH stimulation in normal tissue, availability of precursor compounds was limiting. Evidence from the work of others, then, is consistent with our idea that exogenous squalene acts *in vivo* to augment ACTH action because squalene serves as a readily available precursor of glucocorticoids. Our findings, in turn, can be interpreted as indicating that mechanisms of ACTH action observed *in vitro* also function *in vivo*.

Tentative formulation of the relationship between the effect of ACTH alone and its augmentation by squalene becomes possible when thymic re-

gression data from different experiments are made comparable by conversion to per cent decrease in relative thymus weights and graphed as in Figure 2. Because thymic regression responses to ACTH have been only roughly reproducible on the basis of dosage, the per cent decrease in relative thymus weight in response to ACTH has been substituted for dosage on the abscissae. Per cent decreases in relative thymus weight attributable to squalene, when both ACTH and squalene were given, have been plotted as ordinates.

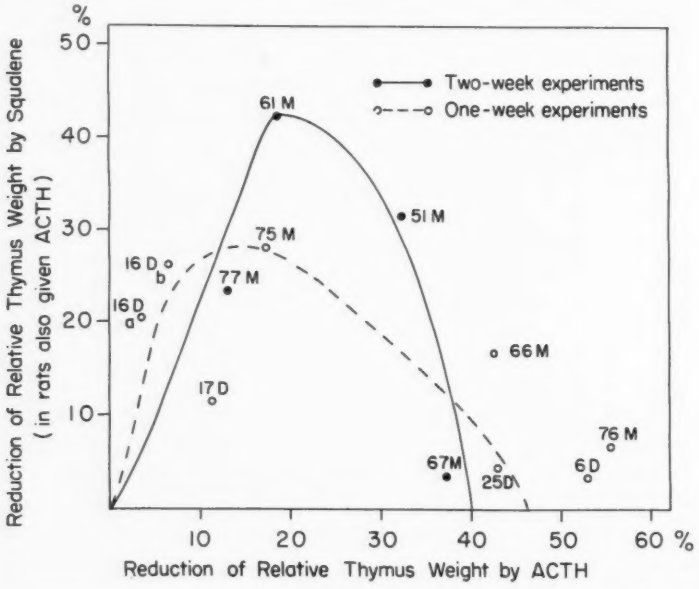


FIG. 2. Relationship of reduction in relative thymus weight by ACTH alone to reduction in relative thymus weight by squalene when this hydrocarbon is administered to ACTH-treated, hypophysectomized rats.

Experiments of the two main types used thus far have been graphed (Fig. 1) for purposes of discussion. Many more data would be required to permit statistically reliable curves to be drawn. The data available allow only the approximations shown. In the two-week experiments, peak augmentation by squalene occurred when ACTH alone produced approximately 20 to 25% decrease in relative thymus weight, whereas in the one-week experiments, maximal potentiation was observed when ACTH alone did not have significant involuting effect. If local absorption of squalene at depot sites and its uptake by the adrenal be the same for all experiments of the same duration, the implications of these curves are that low "doses" of ACTH do not permit maximal conversion of available squalene to gluco-

corticoids and that, beyond the point of maximal augmentation, the greater the effect of ACTH alone, the less is the utilization of squalene in corticoid biogenesis. If, as the dose of ACTH is increased, the amount of squalene available to the adrenal increases (as might be possible for example on the basis of increased blood supply to the gland), the maximum augmentation would occur at the intersection of the regression lines of increasing squalene supply to the adrenal and decreasing squalene utilization in corticoid biogenesis. The positive slope of the curve of squalene supply would necessarily have to be smaller than the negative slope of the curve of squalene disappearance due to utilization.

It seems more probable that the curves in Figure 2 are the resultants or the algebraic sum of many variables. Some of these variables are determinants of the rates of glucocorticoid synthesis, others of glucocorticoid release, transport, destruction or action, and still others, of thymic responsiveness. Despite the involvement of all these factors, it is possible to resolve the matter by assuming that the curves represent the action of the effective amounts of glucocorticoids attributable to squalene converted. On this basis, the ascending limbs of the curves are in accord with hypothesis. That the inflection of the curves and the descending limbs represent a decreased utilization of squalene is more difficult of acceptance.

An alternative interpretation is possible. Decrease in relative thymus weight has been shown to be linear to the log of the dose of glucocorticoid (4, 5) or ACTH (10). That is, production of each successive equal decrement of thymus weight requires a ten-fold increase in hormone administered or produced. That the curves (Fig. 2) do not level off at maximal augmentation but instead decrease to zero as the dose of ACTH is increased further probably indicates that, although the amount of glucocorticoid derived from squalene remains relatively constant, it constitutes a diminishing fraction of the increasing total glucocorticoid production. This would explain why significant augmentation has not occurred at the higher "doses" of ACTH. That is, augmentation decreases as squalene becomes limiting. Pursuing the argument further leads to the prediction that increase in the effectively absorbed dose of squalene will provide for measurable augmentation of higher "doses" of ACTH, within the limits of the thymus assay.

It may be possible to estimate the amounts of glucocorticoid attributable to different doses of squalene administered with each of several doses of ACTH by means of reference to a simultaneously determined dose response curve for a glucocorticoid. Within the limits of experimental error, the amounts of glucocorticoids thus estimated to arise by synthesis from any one dose of squalene should be constant or increased with increasing doses of ACTH. This hypothesis is now being tested experimentally.

Definitive squalene augmentation of ACTH-induced thymic regression in intact rats has not occurred in six experiments with doses of ACTH

which decreased relative thymus weight from 5 to 50%. In several experiments thymus regression in response to ACTH and squalene appeared to be greater than to ACTH alone, but the increased response was not statistically significant. On one occasion, when ACTH caused a 5.5% reduction in relative thymus weight, not a statistically significant effect, the decrease which occurred in response to both ACTH and squalene was statistically significant. It appears that exogenous squalene may be utilized as a precursor of adrenal corticoids in intact rats but that preconditioning by endogenous glucocorticoids makes thymic involution an even more insentitive measurement than it is in hypophysectomized rats. The normal rats used have had a mean relative thymus weight of approximately 250 mg. in contrast to the 325 mg. average weight found in the hypophysectomized rats of the same age, a 23% difference. In addition, augmentation can most easily be demonstrated at a low dosage level of ACTH, a level at which compensation by homeostatic mechanisms would occur in intact rats. A more sensitive technique will be required to determine whether squalene is utilized by intact rats as readily as it appears to be by hypophysectomized rats.

Acknowledgments

The author is grateful to Dr. Richmond W. Smith, Jr., for his interest in this work and for critical reading of the manuscript. Competent technical assistance was given by Messrs. Donald Nemec, James Dilhoeffer, Joseph Sciarroto and Jerome Dudley in Cleveland and by Miss Jalileh Mansour, Miss Lillian Arakelian and Mr. Hakehill Chan in Detroit.

REFERENCES

1. Kline, I. T.: *Endocrinology* **61**: 85. 1957.
2. Langdon, R. G. and K. Bloch: *J. Biol. Chem.* **200**: 135. 1953.
3. Bucher, N. L. R. and K. McGarrahan: *J. Biol. Chem.* **222**: 1. 1956.
4. Santisteban, G. A. and T. F. Dougherty: *Endocrinology* **54**: 130. 1954.
5. Stephenson, N. R.: *Canad. J. Biochem. and Physiol.* **32**: 689. 1954.
6. Dougherty, T. F.: *Physiol. Rev.* **32**: 379. 1952.
7. Ingle, D. J.: *Acta Endocrinol.* **17**: 172. 1954.
8. Selye, H.: "Second Annual Report on Stress," Second ed. Grune & Stratton Company, New York, 1952.
9. Herlant, M.: *Proc. Soc. Exper. Biol. and Med.* **73**: 399. 1950.
10. Thompson, R. E. and J. D. Fisher: *Endocrinology* **52**: 496. 1953.
11. Langdon, R. G. and K. Bloch: *J. Biol. Chem.* **200**: 135. 1953.
12. Loud, A. V.: *Fed. Proc.* **15**: 122. 1956.
13. Haynes, R. C., Jr. and L. Berthet: *J. Biol. Chem.* **225**: 115. 1957.
14. Koritz, S. B. and D. W. Peron: *J. Biol. Chem.* **230**: 343. 1958.

THE DIABETIC RESPONSE OF GEESE TO PANCREATECTOMY[1]

I. ARTHUR MIRSKY AND SIMON GITELSON[2]

Department of Clinical Science, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

ABSTRACT

Removal of the pancreas together with the gastrointestinal tract results in a marked impairment in the utilization of glucose and in an inhibition of the hypoglycemic response to tolbutamide in the goose but not in the duck.

THE utilization of carbohydrates does not appear to be impaired significantly in the depancreatized chicken or duck (1, 2, 3, 4, 5, 6). Likewise these birds show no impairment in the hypoglycemic response to tolbutamide after pancreatectomy (7). The present report deals with studies which reveal that unlike the chicken or duck, the goose develops a diabetic syndrome in the absence of the pancreas.

METHODS

Domestic ducks (Pekin strain) and geese (Emden and Toulouse strains) of from 2 to 5 kilograms body weight were used in this study. The blood sugar response to the intravenous injection of 0.1 unit insulin per kilogram body weight was determined before pancreatectomy. The response to the intravenous injection of 1.75 gm. glucose per kg. body weight was determined before and after removal of the pancreas as was also the response of another group of ducks and geese to 50 mg. tolbutamide per kg. body weight. Before and after the intravenous injection, samples of venous blood were taken at intervals from an exposed wing vein or from an exposed jugular vein. The concentration of glucose in the blood was determined by the Nelson procedure (8).

In order to ensure the complete removal of the pancreas and of any accessory pancreatic tissue, the pancreas was removed together with the entire gastrointestinal tract from the esophagus to the rectum. The cloaca was left intact in the rectal stump in order to allow the free passage of urine. Care was taken to avoid tying the hepatic artery; its patency and continuity from its origin in the celiac artery and posterior aorta to its entrance into the liver was established by post-mortem examination. The enterectomy was performed under ether anesthesia which permitted the fowl to recover rapidly after the termination of the operation. Such birds have survived as long as 24 hours.

RESULTS AND DISCUSSION

That the metabolism of carbohydrate is essentially the same in the normal duck and goose is suggested by their responses to the administration of insulin (Fig. 1) and of glucose (Fig. 2). Although the normal duck devel-

Received April 3, 1958.

[1] Aided by a grant from the U. S. Public Health Service (A-1409).

[2] Aided by the Emanuel Spector Memorial Foundation.

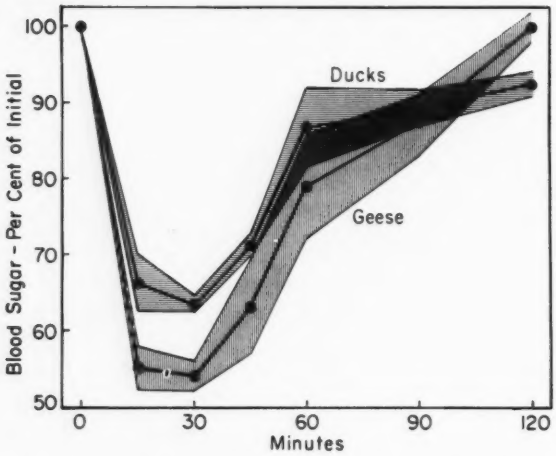


FIG. 1. The responsiveness of normal ducks and geese to insulin. Each curve represents the mean (●) ± S.E. (shaded area) of the blood sugar concentration of six birds expressed as the per cent of that before the injection of 0.1 unit insulin per kilogram body weight.

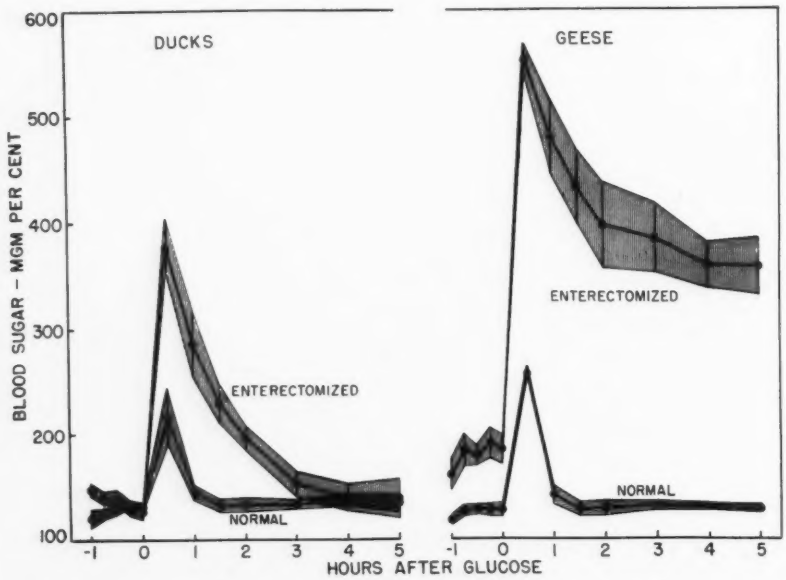


FIG. 2. The glucose tolerance of ducks and geese before and after removal of the pancreas. Each curve represents the mean (●) ± S.E. (shaded area) blood sugar concentration of six birds for an hour before and five hours after the injection of 1.75 grams glucose per kilogram body weight. The enterectomy was completed one hour before the injection of the glucose.

oped a smaller hypoglycemic response during the first thirty minutes after he exogenous insulin, the overall response is essentially the same in both species.

Immediately after the removal of the pancreas together with the gastrointestinal tract, the blood sugar concentration increased from 131 ± 3.9 to 148 ± 6.8 mg. % in the ducks and from 128.2 ± 5.9 to 161.4 ± 13.6 mg. % in the geese. At the end of the first hour after the enterectomy, however, the blood sugar of the ducks was back to the preoperative level of 126.8 ± 12 mg. % while that of the geese rose to 183.8 ± 14.4 mg. %.

The effect of the injection of 1.75 gm. glucose per kg. body weight on the blood sugar concentration of enterectomized ducks and geese is depicted in Figure 2. It is apparent that the enterectomy produced a decrease in the rate of glucose removal from the blood of both the ducks and geese. In the ducks, however, the blood sugar returned to the pre-injection level within 3 hours while in the geese the hyperglycemia was much greater and persisted for more than 5 hours. In some instances the geese were not sacrificed before 24 hours after the enterectomy at which time they still exhibited a marked hyperglycemia.

The effect of the intravenous injection of 50 mg. tolbutamide per kilo-

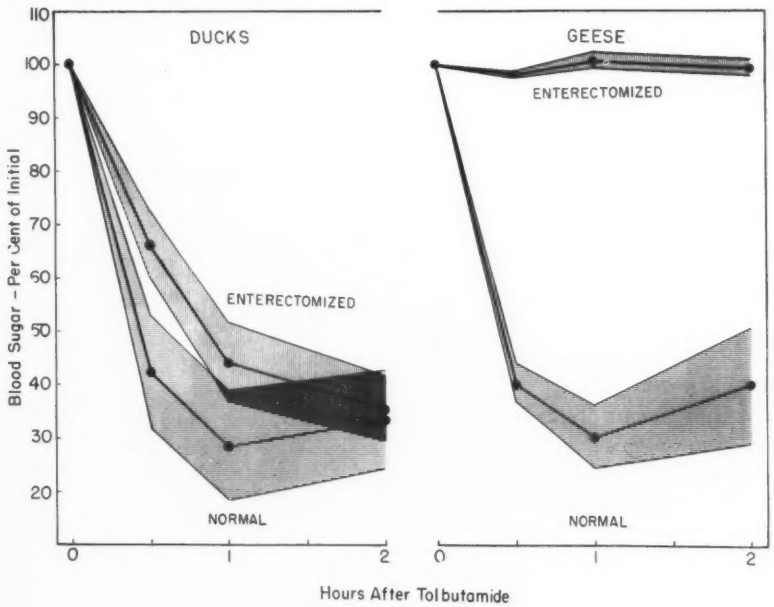


FIG. 3. The response of normal and depancreatized ducks and geese to tolbutamide. Each curve represents the mean (●) ± S.E. (shaded area) blood sugar concentration of six birds expressed as the per cent of that before the injection of 50 mg. tolbutamide per kilogram body weight.

gram body weight on the blood sugar of normal and enterectomized ducks and geese is illustrated in Figure 3. Both normal ducks and geese responded to the tolbutamide with a significant decrease in the blood sugar concentration. Removal of the pancreas resulted in no significant impairment in the hypoglycemic response of the duck. The enterectomized goose, however, developed no hypoglycemic response to the injection of tolbutamide.

These data are in accord with earlier observations that removal of the pancreas from the duck produced no significant impairment in the utilization of injected glucose (6) or in the hypoglycemic response to tolbutamide (7) other than that attributable to the anesthesia and the trauma of the operation. The same procedure in the goose resulted in an impairment in glucose utilization and in the hypoglycemic response to tolbutamide. Accordingly, the goose behaves in this respect like most mammals.

Whereas the depancreatized chicken and duck do not develop the metabolic derangement of diabetes mellitus, removal of the pancreas from the hawk and owl (2, 3, 9) results in a marked impairment in carbohydrate metabolism. Such observations prompted the hypothesis that the innate food habits of the species played some role in determining the susceptibility to the diabetic syndrome. The data reported herein cast much doubt upon the validity of this hypothesis since the goose, an herbivorous bird, develops a marked impairment in the utilization of glucose after removal of the pancreas.

REFERENCES

1. LANGENDORF, O.: *Arch. f. Anat.*, Leipz, pp. 1–35. 1879.
2. MINKOWSKI, O.: *Arch. f. exp. Path. u. Pharmakol.* **31:** 85. 1892.
3. WEINTRAUD, W.: *Arch. f. exp. Path. u. Pharmakol.* **34:** 303. 1894.
4. KAUSCH, W.: *Arch. f. exp. Path. u. Pharmakol.* **37:** 274. 1896.
5. SEITZ, I. J. AND A. C. IVY: *Proc. Soc. Exp. Biol. and Med.* **26:** 463. 1929.
6. MIRSKY, I. A., N. NELSON, I. GRAYMAN AND M. KORENBERG: *Am. J. Physiol.* **135:** 223. 1941.
7. MIRSKY, I. A. AND S. GITELSON: *Endocrinology* **61:** 148. 1957.
8. NELSON, N.: *J. Biol. Chem.* **153:** 375. 1944.
9. NELSON, N., S. ELGART AND I. A. MIRSKY: *Endocrinology* **31:** 119. 1942.

MEASUREMENT OF FREE CORTICOSTEROIDS IN RAT PLASMA: PHYSIOLOGICAL VALIDATION OF A METHOD[1]

ROGER GUILLEMIN, GEORGE W. CLAYTON, J. D. SMITH AND HARRY S. LIPSCOMB[2]

Departments of Physiology and Pediatrics, Baylor University College of Medicine, Houston, Texas

ABSTRACT

Levels of free plasma corticosteroids (expressed as μg. Compound B/100 ml. plasma) have been measured routinely in 2 ml. of rat plasma using a method based on fluorescence of Compound B in H_2SO_4. Resting levels are 9 to 15 μg./100 ml. After stress, levels increase up to 500%. After total adrenalectomy or hypophysectomy readings correspond to 3.5–6.5 μg./100 ml. There is no change upon exposure to stress of the adrenalectomized or hypophysectomized animal. Intra-venous injection of 0.1 to 1.0 milliunit of ACTH U.S.P. Standard, 24 hours after hypophysectomy increases the concentrations of corticoids in a linear log-dose response. After adrenalectomy ACTH is without effect. In the hypophysectomized animals, large doses of highly purified FSH, LH, TSH, STH, α-MSH and arginine vasopressin have no effect on the corticoid levels. Considerable stimulation of corticoidogenesis can be observed in the absence of detectable changes in the adrenal ascorbic acid concentrations when the two variables are measured simultaneously in the same hypophysectomized animals.

A SIMPLE method based on the fluorescence of corticosterone in sulfuric acid was recently devised by Silber to measure levels of "free" corticosteroids in small volumes of plasma (1). This report is a summary of experiments performed to assess the validity of this method in various experimental conditions. Two preliminary notes on some of these data have been published elsewhere (2, 3).

MATERIALS AND METHODS

1. *Animals.* Approximately 700 animals were used in the experiments reported here; they were male rats, weighing 160–210 gm. obtained from Holtzman Farms in Houston and Madison. Females were used in a series of experiments devoted to a study of the relationships between the ovarian cycle and the resting levels of plasma corticosteroids; stages of the cycle (proestrus, estrus, diestrus) were assessed for 7 days before measurement of corticosteroid levels by daily vaginal smears stained with 1% toluidine blue.

Received April 22, 1958.

[1] These experiments were supported by funds provided under Contract No. AF 41(657)-17 with the USAF School of Aviation Medicine, Randolph Field, Texas and by Grant No. A-1276 from the U. S. Public Health Service.

[2] Daland Fellow of the American Philosophical Society.

Bilateral adrenalectomies were performed through lumbar incisions under ether anesthesia; the animals were maintained with 0.9% NaCl as drinking fluid; some received desoxycorticosterone acetate s.c. as a suspension in oil at a dose of 50 μg./day. Several groups were also castrated at the time of bilateral adrenalectomy. Hypophysectomies were performed by Hormone Assay Laboratories, Chicago; the animals were received 12 to 15 hours after operation.

2. *Stressing procedures.* Several types of stress were used: exposure of the left common carotid artery followed by injection of 0.2 ml. NaCl 0.9% and tying of the vessel 1 minutes after injection of pentobarbital (4.5 mg./100 gm. B.W., i.p.); bilateral fracture of tibia and fibula (the animals received pentobarbital as above at the same time, anesthesia being so induced in approximately 4 minutes); the intraperitoneal injection of pentobarbital considered as a minor sensory stress was used in several experiments.

3. *Sampling of blood.* Several methods were used for obtaining "resting levels" of corticosteroids. Blood was collected (a) in heparinized beakers from the trunk of rats rapidly decapitated in the animal room; (b) in heparinized syringes from the jugular vein in rats given pentobarbital (8 mg./100 gm. B.W., s.c.) 30 to 40 minutes previously; (c) from the abdominal aorta in rats given pentobarbital (4.5 mg./100 gm. B.W., i.p.) 30 to 40 minutes previously; (d) from the abdominal aorta of rats given pentobarbital (as in c) at time zero, morphine sulfate (i.p., 2.0 mg./100 gm. B.W.) at +25 minutes, blood being sampled at +55 minutes. All animals given pentobarbital and morphine were kept on an electric blanket maintained at 38° C under a lucite box in an atmosphere of 95% O_2, 5% CO_2. For procedures (c) and (d) the total time for sampling the blood i.e. from opening the skin to withdrawing from the aorta the 5 ml. syringe full of blood, must be $\leqq$40 seconds. This last procedure was adopted for all "experimental" animals after having been assessed in a series of "controls" for obtaining resting levels of corticosteroids. Hypophysectomized or adrenalectomized animals were bled from the abdominal aorta under ether or pentobarbital anesthesia. All blood samplings were done between 7:00 A.M. and 12:00 A.M.

Within 90 seconds after withdrawal, blood samples were centrifuged and the plasma separated. Plasma samples were then kept frozen.

4. *Measurement of the plasma corticosteroids and adrenal ascorbic acid concentrations.* Determination of the plasma free corticosteroids was carried out on 2 ml. aliquots of plasma according to the method of Silber (1) the main modification being that a Farrand (Model A) fluorometer was used with the filter system suggested by Sweat (4).

In several experiments, pooled samples of rat plasma were extracted according to the above method and chromatographed in the E2B system of Eberlein (5). Adrenal ascorbic acid concentrations were measured by the method of Roe and Kuether (6) in which the 4 hour incubation period at 37° C was replaced by a 15 minute incubation in boiling water.

5. *Drugs and hormonal preparations.* Two preparations of heparin were used: Liquaemin Sodium, 1000 USP U./ml. (Organon, Inc.) and Heparin Sodium, 10,000 USP U./ml. (Abbott Laboratories). Pentobarbital sodium, as crystalline Nembutal (Abbott Laboratories) was freshly dissolved in 0.9% NaCl solution before each experiment. Epinephrine soluble salt, 1 mg. tablets (The Upjohn Co.), *d-l* norepinephrine HCl (Winthrop-Stearns Inc.), 5-hydroxytryptamine as serotonine creatinine sulfate (The Lilly Research Laboratories) were dissolved in aliquots of plasma from their crystalline forms for various experiments.

U.S.P. Corticotropin Reference Standard (1.14 U./mg.) was used exclusively in all experiments where effects of ACTH were studied. Hence ACTH as used in this text will refer to the present USP Reference Standard for Corticotropin. Highly purified arginine-vasopressin (380 U./mg.) had been prepared by the method of Ward and Guillemin (7).

Growth hormone (STH, 1.3 I.U./mg.) of bovine origin had been prepared by a modification of the method of Wilhelmi and Fishman (8). Follicle stimulating hormone, (FSH, 30 × Armour Standard) of porcine origin had been prepared by the method of Steelman *et al.* (9). Luteinizing hormone (LH, 1 × Armour Standard) of ovine origin was prepared by the method of Ward (10). Thyrotrophic hormone (TSH, 1.0 U./mg.) of bovine origin had been prepared by the method of Condliffe (11). α-MSH of porcine origin had been prepared by the method of Steelman (12).

EXPERIMENTAL

The method for determining plasma corticoids as utilized here detects easily 0.02 μg. of corticosterone with recoveries from water of 105.0% ± 1.2 and from plasma of 100.3% ± 0.4 at concentrations ranging from 0.1 μg./ml. to 2.0 μg./ml. (Table 1 A). These results confirm the data obtained by Silber (1). Pools of plasma were prepared and distributed in 2.2 ml. aliquots and kept frozen for subsequent determinations to check reproducibility of the method. The reproducibility of the results obtained in a given sample is excellent and it would appear that there is no appreciable variation of the corticosteroid concentration with storage of the plasma in the frozen state (Table 1B).

Chromatographic analysis of the chloroform extract of pooled rat plasma revealed only one spot having the mobility of corticosterone. In several experiments the amount of corticosterone in this area was determined by fluorometry. It was $\geq$91% of the value for "corticosteroids" as measured in the original plasma sample by the procedure used throughout this paper.

Heparin does not appear to interfere with the measurement of corticosterone by this method: measurements on sera obtained because of accidental omission of heparin have not shown any difference from those on plasma within the same group. Injection of Nembutal, or morphine sulfate does not lead to interfering fluorescence with the method as used here (Table 2).

Addition to plasma of crystalline epinephrine, norepinephrine and serotonine creatinine sulfate in doses at least 1000 times larger than those found in experimental situations where these substances are known to be at maximal levels (13, 14) does not interfere with the fluorometric measurement of corticosterone (Table 1 C).

TABLE 1

		Number of experiments	Corticosterone, amount added	% recovery ± S.E.
A	Recoveries from Water	61	0.1 to 2.0 μg.	105.0 ± 1.2
	Recoveries from Plasma	16	0.8 to 1.0 μg.	100.3 ± 0.4
		Number of experiments	Corticosterone, μg./100 ml. ± S.E.	Total storage time
B	Plasma Pool #A	11	16.8 ± 0.60	14 days
	#B	3	18.3 ± 0.78	3 days
	#C	7	27.0 ± 0.33	55 days
	#D	27	12.5 ± 0.29	105 days
	#E	11	20.8 ± 0.35	60 days

		Corticosterone μg./100 ml.		
C	Plasma Sample	18.2	17.2	19.1
	+ Epinephrine 0.9 mg./100 ml.	16.2	17.2	
	+ Nor-epinephrine 1.0 mg./100 ml.	19.1	17.2	
	+ Serotonine 0.86 mg./100 ml.			20.2

In the following experiments the data reported will therefore indicate levels of plasma free corticosteroids expressed as micrograms of corticosterone per 100 ml. of plasma.

RESULTS AND COMMENTS

With the method as used in this laboratory, utilizing animals of this strain and of this weight range, *basal* or *resting* levels of plasma free corticosteroids are from 9.0 to 15.0 μg./100 ml. (mean and standard error: 12.2 ±0.30 for 128 determinations. Table 2). In a small series of animals no variation of plasma corticoid levels was observed in relation to the phases of the ovarian cycle (Table 2 C).

TABLE 2. RESTING LEVELS OF PLASMA CORTICOSTEROIDS IN THE RAT IN μG. OF CORTICOSTERONE/100 ML. PLASMA

Mode of blood sampling		Remarks	Number of animals	Cpd. B, μg./100 ml. plasma
A	Decapitation	a	12	15.2 ±1.9*
B	Jugular Vein	b	31	14.2 ±0.48
	Abdominal Aorta	c	30	11.5 ±0.73
	Abdominal Aorta	d	14	11.0 ±0.40
	Abdominal Aorta	e	47	12.7 ±0.56
C	Abdominal Aorta	c		
	Proestrus		4	11.4 ±1.0
	Estrus		5	10.1 ±0.39
	Diestrus		16	10.3 ±0.38

Remarks: See in METHODS for details. a: Decapitation in animal room. b: Sub-cutaneous Nembutal. c: Intraperitoneal Nembutal. d: Nembutal + Morphine. e: Nembutal + Morphine +0.2 ml. Saline i.v.

* Standard error of the mean.

It was observed that changing the environment of the animals by taking them from animal quarters to the laboratory increases the level of the corticoids within a few minutes. A group of 18 normal rats was left for 15–20 minutes in the laboratory while other animals were being sacrificed; when they were bled by decapitation their plasma corticosteroid level was 40.6 ±2.5. This emphasizes that the utmost care must be taken for obtaining adequate "resting" control levels.

After bilateral adrenalectomy or hypophysectomy this resting level falls to 3.5 to 6.5 μg./100 ml. This figure does not vary with increasing intervals after adrenalectomy or hypophysectomy; after combined adrenalectomy and gonadectomy; upon exposure to stress of the adrenalectomized or hypophysectomized animal or upon intravenous injection of massive doses of ACTH in the adrenalectomized animal (Table 3). This *residual fluorescence* is therefore not due to corticoids of adrenal origin but to some non-identified substances which are not related to pituitary-adrenal stimula-

TABLE 3. PLASMA CORTICOSTEROID LEVELS IN μG./100 ML. PLASMA AND READINGS OF RESIDUAL FLUORESCENCE AT TIME OR DATE SHOWN AFTER PROCEDURE

Procedure	No. of animals per exp.	15 min.	30 min.	60 min.	24 hrs.	48 hrs.	3 days	4 days	5 days	10 days	15 days	30 days
Bilateral Adrenalectomy	36	17.5±0.6*	15.1±1.4	9.7±0.2	—	—	6.1±0.4	—	5.6±0.5	6.7±0.5	6.0±0.7	—
Bilateral Adrenalectomy & Gonadectomy	24	—	—	—	—	—	—	—	7.6±0.4	5.2±0.2	5.5±0.5	—
Bilateral Adrenalectomy+DOCA	14	—	—	—	—	—	—	—	5.6±0.5	6.3±0.2	5.6±0.6	—
Bilateral Adrenalectomy+Gonadectomy+DOCA	17	—	—	—	—	—	—	—	5.7±0.5	6.3±0.6	5.1±0.4	—
Hypophysectomy	62	—	—	—	6.9±0.4	7.0±0.6	—	6.1±0.5	—	6.5±0.3	5.6±0.8	5.3±0.5
Bilateral Adrenalectomy+ACTH	6	15 minutes after 5.0 mu ACTH i.v.			—	—	—	—	5.2±0.3	—	—	—
Bilateral Adrenalectomy+Stress	5	15 minutes after carotid cannulation			—	—	—	—	6.0±0.8	—	—	—
Bilateral Adrenalectomy+Gonadectomy+Stress	9	15 minutes after carotid cannulation			—	—	—	—	6.3±0.2	5.7±0.2	—	—
Hypophysectomy+Stress	7	15 minutes after carotid cannulation			5.4±0.2	—	—	—	—	—	—	—

* Standard error of the mean.

tion. The value of this residual fluorescence is most constant within any given group of adrenalectomized or hypophysectomized animals. Because its variations, though minimal, are definite between groups (see Table 3) it was thought best to keep this background fluorescence into the calculations of corticosteroid concentrations.[3] If and when means are found to eliminate this residual fluorescence, the absolute values as well as the percentage variations of these and similar measurements can be expected to be appreciably modified. This should not alter the conclusions relative to the validity of the method as presently employed.

Exposure of the normal animal to stress of systemic type elevates the levels of the plasma corticosteroids. The maximal concentration was observed approximately 15 minutes after beginning of stress (Table 4), when

TABLE 4. PLASMA FREE CORTICOSTEROIDS IN THE NORMAL RAT UPON EXPOSURE TO STRESS IN μG. CORTICOSTERONE/100 ML.

Procedure	Number of animals per experiment	Time after beginning of stress					
		5 min.	10 min.	15 min.	30 min.	45 min.	60 min.
Cannulation of Carotid Artery	28	33.5±1.0*	38.8±0.9	46.5±2.5	37.6±3.6	34.2±3.2	—
Bilateral Fracture of Tibia and Fibula	35	29.9±1.6	36.7±2.5	48.1±2.8	53.1±1.8	—	41.6±4.7
		4 min.	8 min.	12 min.	16 min.	20 min.	24 min.
Nembutal i.p.	65	16.2±1.2	12.2±1.4	19.6±1.5	20.3±1.9	21.7±1.5	23.4±1.2
					28 min.	32 min.	35 min.
					15.9±1.0	16.8±1.3	10.4±0.6

* Standard error of the mean.

the intervals studied were 5, 10, 15, 30, 45 and 60 minutes. This high level persists or tends to decrease within 60 minutes of observation, apparently in relation to the severity of the stress applied. Sensory or emotional stress also elevates the level of plasma corticoids: handling and intraperitoneal injection of Nembutal in saline produced a minimal but definite increase of the plasma corticoid concentration which returned to normal in approximately 30 minutes (Table 4).

Intra-venous injection of ACTH (0.05 to 1.0 milliunit) in animals 24–27 hours after hypophysectomy increased the levels of plasma free corticosteroids; the maximal levels were found 15 minutes after injection when time intervals studied were 15, 30 and 60 minutes (Table 5A). This time relationship was confirmed when the plasma corticosteroid levels were measured at 2 minute intervals after injection of 0.3 milliunit ACTH (Table 5B). When the plasma corticoids are measured 15 minutes after injection of ACTH the limit of sensitivity of the method is $\leqq$0.1 milliunit U.S.P. Reference Standard for Corticotropin. In several experiments when doses

[3] A calculation has recently been proposed by Silber *et al.* (See reference no. 1) to exclude this "residual fluorescence" from the readings.

TABLE 5. CONCENTRATIONS OF PLASMA FREE CORTICOSTEROIDS AND OF ADRENAL ASCORBIC ACID IN THE HYPOPHYSECTOMIZED RAT AFTER INJECTION OF ACTH. A AND B: AS A FUNCTION OF TIME AFTER INJECTION. C: AS A FUNCTION OF THE DOSE

	Time in minutes	ACTH, mU.	A.A.A.* μg./100 mg.	Cpd. B, μg./100 ml. plasma	A.A.A. variation, % controls	Cpd. B variation, % controls
A	0	None	474 ± 12†	6.2 ± 0.3	—	—
	15	0.25	412 ± 26	19.1 ± 1.7	−13	+208
	30	0.25	427 ± 14	10.4 ± 1.0	− 9	+ 67
	60	0.25	415 ± 6	6.5 ± 0.6	−12	+ 4
	15	0.50	365 ± 5	38.3 ± 3.4	−22	+518
	30	0.50	367 ± 5	22.5 ± 1.7	−22	+263
	60	0.50	384 ± 16	8.0 ± 0.4	−19	+ 29
	0	None	469 ± 18	6.3 ± 0.5	—	—
	15	1.0	309 ± 8	45.5 ± 1.6	−34	+622
	30	1.0	312 ± 5	40.5 ± 2.4	−33	+542
	60	1.0	321 ± 23	23.6 ± 2.5	−31	+274
B	0	None	481 ± 19	5.6 ± 0.2	—	—
	2	0.3	473 ± 15	5.4 ± 0.3	− 1	—
	4	0.3	464 ± 13	8.0 ± 0.6	− 3	+ 43
	6	0.3	458 ± 12	12.8 ± 2.8	− 4	+128
	8	0.3	411 ± 12	19.3 ± 0.9	−14	+244
	10	0.3	461 ± 31	20.7 ± 1.7	− 4	+273
	12	0.3	463 ± 10	25.4 ± 2.6	− 4	+353
	15	0.3	460 ± 17	34.5 ± 0.9	− 4	+516
	20	0.3	485 ± 31	22.2 ± 0.7	+ 1	+296
C	0	None	472 ± 10	6.4 ± 0.4	—	—
	15	0.05	503 ± 6	7.3 ± 0.3	—	+ 14
	15	0.1	485 ± 13	8.7 ± 0.9	—	+ 36
	15	0.2	474 ± 15	12.4 ± 1.6	—	+ 94
	15	0.4	408 ± 13	27.8 ± 1.8	−13	+334
	15	0.8	357 ± 12	38.2 ± 2.3	−32	+497

* A.A.A. = adrenal ascorbic acid. A, B, and C were three different experiments on three different shipments of animals. A and C, six animals per group; B, four animals per group; 8 to 10 animals in control groups.

† Standard error of the mean.

of 0.05 to 1.0 mU. ACTH were administered, the responses obtained appeared as a linear function of the log of the dose between 0.2 and 1.0 mU. (Tables 5A, 5B, 5C, Fig. 1). The linear relation was observed when the corticoids were measured at 15, 30 or 60 minutes after injection of ACTH. A complete evaluation of the limits of the response to ACTH was not done within this series of experiments.

Injection of large doses of purified TSH, STH, FSH, LH, α-MSH and arginine-vasopressin in the hypophysectomized rat failed to increase the level of corticosteroids in experiments designed to demonstrate the effects of ≦0.1 mU. ACTH (Table 6), with measurement of the response 15 minutes after injection.

Tables 5A, 5B and 5C show that the response to ACTH is remarkably constant within a given group (shipment) of animals but that it varies slightly from one shipment to another. This is probably another example of the variations of sensitivity of the animal preparation, a well known characteristic inherent to bio-assays.

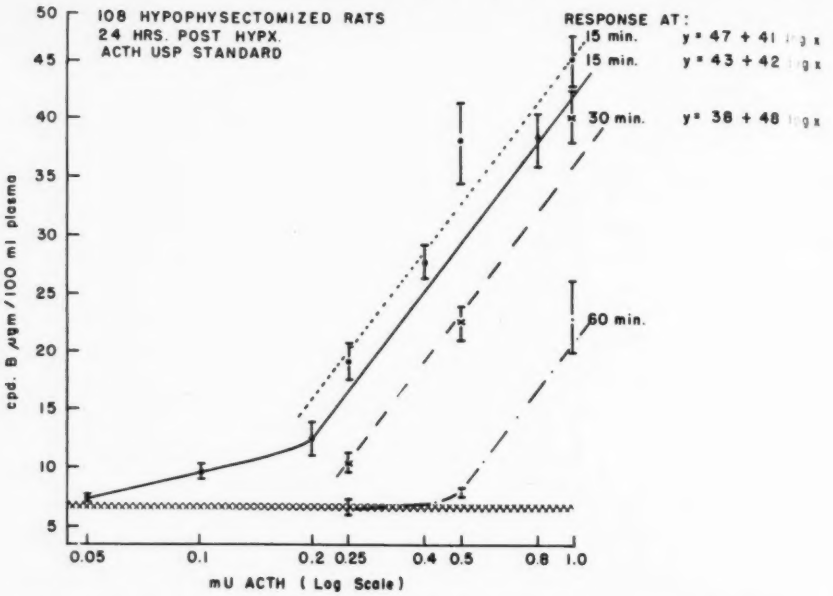


FIG. 1. Data of table 5 calculated to show the linearity of the response when expressed as a function of the $\log_{10}$ of the dose of ACTH injected. $Y = a + b \log x$, for $0.2 \leqq x \leqq 1.0$.

It is interesting to note that the time relationships for adrenal response to ACTH are close to those observed for pituitary-adrenal response to stress (Tables 4 and 5). The time relationships for adrenal response to ACTH as observed here in the hypophysectomized rat are in remarkable agreement with those reported by Nelson and Hume in the hypophysectomized dog (15) using measurements of the corticoid output in the adrenal vein but are at variance with those reported recently by Slusher and

TABLE 6. PLASMA CORTICOSTEROID LEVELS IN HYPOPHYSECTOMIZED RATS 15 MINUTES AFTER INJECTION OF VARIOUS HORMONAL PREPARATIONS OF PITUITARY ORIGIN OTHER THAN ACTH

Material injected	Amount injected	Number of animals	Cpd. B, µg./100 ml. plasma
Saline	0.3 ml.	4	4.4 ± 0.3*
STH	7.1 µg.	4	4.9 ± 0.3
FSH	4.8 µg.	4	5.8 ± 0.7
LH	5.0 µg.	4	6.7 ± 1.0
TSH	2.4 µg.	4	5.7 ± 0.5
Arginine-Vasopressin	0.8 µg. (300 mU.)	3	6.9 ± 0.5
Saline	0.3 ml.	4	7.1 ± 0.6
MSH	100 µg.	4	6.9 ± 0.6

* Standard error of the mean.

Roberts in similar experiments using rats (16). These time relationships between ACTH-administration and the observed maximal level of circulating free corticosteroids (ca. 15 minutes) exclude the possibility that we may be dealing in this series of experiments with an extra adrenal action of ACTH which would tend to inhibit conjugation of the free corticoids. Conjugation takes place in an entirely different chronological relation following injection of ACTH (17).

In some of these experiments simultaneous measurements of adrenal ascorbic acid concentrations showed that considerable stimulation of corticoidogenesis can be obtained in absence of detectable ascorbic acid depletion (Tables 5A, 5B, 5C). When a dose of ACTH is injected which is large enough to *significantly* decrease the concentration of adrenal ascorbic acid, the fall in the adrenal content of this substance reaches its maximum around 15 minutes later, and remains at that level for 1 hour (Table 5A) or more (18). Meanwhile, considerable dynamic changes of the levels of the circulating corticoids can be demonstrated (Tables 5A and 5B).

Within this limited series of experiments, the fluorometric method of Silber for corticosterone was found to be simple enough for routine use. It appears reasonably specific for studying in the plasma the dynamic variations of substances of adrenal origin of the corticosterone type. It is of a degree of sensitivity such as to permit measurements well within physiological requisites. The reproducibility of the results is good both within groups and between groups. The method therefore may be an ideal tool in studies of the physiology of the hypothalamo-pituitary-adrenal system in these species in which the main product of the adrenal secretion is corticosterone. It may also constitute the basis of a bio-assay for ACTH less indirect than the present provisional assay.

Acknowledgments

We would like to express our particular gratitude to Dr. Robert Silber, Merck Institute for Therapeutic Research, Rahway, N. J., for having communicated to us before publication the analytical method for fluorometric measurement of plasma corticosterone used here.

FSH, STH, α-MSH, were prepared by Dr. S. Steelman, Department of Biochemistry, Baylor University College of Medicine, Houston, Texas; TSH by Dr. P. Condliffe, National Institutes of Health, Bethesda, Md.; LH and arginine vasopressin by and in collaboration with Dr. D. N. Ward, Department of Biochemistry, Baylor University College of Medicine and M.D. Anderson Hospital, University of Texas, Houston, Texas.

The excellent technical assistance of Mrs. Serlina W. Robinson is gratefully acknowledged.

REFERENCES

1. Silber, R. H., R. D. Busch and R. Oslapas: *Clin. Chem.* in press, 1958.
2. Guillemin, R., G. W. Clayton, J. D. Smith and H. S. Lipscomb: *C.R. Acad. Sc.* (Paris) **245**: 1834. 1957.
3. Guillemin, R., G. W. Clayton, J. D. Smith and H. S. Lipscomb: *Fed. Proc.* **17**: 63. 1958.

4. Sweat, G.: *Ann. Chem.* **26:** 773. 1954.
5. Eberlein, W. R. and A. M. Bongiovanni: *Arch. of Biochem. and Biophy.* **9:** 90. 1955.
6. Roe, J. H. and C. A. Kuether: *J. Biol. Chem.* **147:** 399. 1943.
7. Ward, D. N. and R. Guillemin: *Proc. Soc. Exper. Biol. & Med.* **96:** 568. 1957.
8. Wilhelmi, A. E., J. B. Fishman and J. A. Russel: *J. Biol. Chem.* **176:** 735. 1948.
9. Steelman, S. L., T. L. Kelly, A. Segaloff and G. F. Weber: *Endocrinology* **59:** 256. 1956.
10. Ward, D. N. and E. E. New: *Fed. Proc.* **16:** 266. 1957.
11. Condliffe, P. G. and R. W. Bates: *J. Bio. Chem.* **223:** 843. 1956.
12. Steelman, S. L.: Program Endocrine Society, 1958 Meeting, p. 63.
13. Manger, W. M., J. L. Bollman, F. T. Maher and J. Berkson: *Am. J. Physiol.* **190:** 310. 1957.
14. Udenfriend, S., P. A. Schore, D. F. Bogdanski, H. Weissbach, B. Brodie: *Rec. Prog. Horm. Res.* **13:** 1. 1957.
15. Nelson, D. H. and D. M. Hume: *Endocrinology* **57:** 184. 1955.
16. Slusher, M. A. and S. Roberts: *Endocrinology* **61:** 98. 1957.
17. Samuels, L. T., H. Brown, K. Eik-Nes, F. H. Tyler and O. V. Dominguez: *CIBA Foundation Colloquia on Endocrinology* **11:** 208. 1957.
18. Sayers, M. A., G. Sayers and L. Woodbury: *Endocrinology* **42:** 379. 1948.

THE EFFECT OF PROTRACTED GLUCAGON ADMINISTRATION ON BLOOD GLUCOSE AND ON PANCREATIC MORPHOLOGY[1]

SYDNEY S. LAZARUS AND BRUNO W. VOLK
with the technical assistance of PATRICIA LOFARO
Isaac Albert Research Institute of the Jewish Chronic Disease Hospital and the Department of Pathology, Albert Einstein College of Medicine, New York, N. Y.

ABSTRACT

A study was conducted on the effect of long continued administration of glucagon with or without cortisone on blood and urine glucose and on pancreatic morphology in rabbits. One mg. of crystalline glucagon was administered subcutaneously 3 times daily at 8 hour intervals either alone or together with 1 mg./kg. daily of cortisone acetate intramuscularly. Glucagon alone produced only a moderately increased glycemia. On the other hand, glucagon with cortisone produced a marked hyperglycemic status which generally tended to ameliorate after approximately 6 weeks of treatment. Morphologically, there was in most glucagon treated animals an apparent reduction in the numbers of identifiable pancreatic A cells. In many instances partly degranulated or poorly staining A cells were observed as well as groups of nuclei with ill-defined cytoplasm which resembled the nuclei of identifiable A cells and which were thought to be degranulated A cells. The inference from these A cell changes is that glucagon is a hormone and that it is derived from this cellular system. Additional findings included B cell degranulation and glycogen infiltration of duct epithelium and of B cells. Furthermore, in animals treated for prolonged periods there was B cell hyperplasia as well as neogenesis of these cells from ductular epithelium via primitive B cell formation. These latter morphologic findings support the viewpoint that the diminishing diabetogenic effectiveness of these hormones with prolongation of treatment is due to increased pancreatic insulinogenesis.

ALTHOUGH the hyperglycemic action of glucagon is an established fact there are but few studies as to its effect on the structure of the pancreas when given over prolonged periods of time. Kracht (1, 2) reported involution of the pancreatic A cells and degranulation and hypertrophy of the B cells after glucagon administration for 6 to 38 days in the rat and rabbit. He interpreted the atrophy of the A cells as being the result of secretory inactivity, thus, supporting the view that glucagon is produced by this cellular system (3, 4, 5). On the other hand the hypertrophy and degradulation of the B cells were believed to be due to their stimulation by the glucagon induced hyperglycemia. Salter, *et al.* (6) reported a diabetogenic effect of glucagon in rats when up to 1.2 mg., suspended in corn oil,

Received April 22, 1958.
[1] Supported by a grant from Eli Lilly and Co.

was given in divided doses daily, for up to 10 days. They, also produced a transient hyperglycemia and mild glycosuria, in 2 dogs, by daily administration of glucagon, for one week. Upon histologic examination of the pancreas they observed either more intense granulation or degranulation and hydropic degeneration of the B cells as well as marked degranulation and atrophy of the acinar cells, but do not report any A cell changes.

In previous work the authors had demonstrated a potentiating action of cortisone on the hyperglycemic effect of glucagon in the rabbit (7, 8). Furthermore, other workers have shown in chronic experiments that glucagon augments cortisone and adrenocorticotropin induced hyperglycemia in the rat (9). These observations stimulated a study of the effect of long term administration of glucagon given alone or together with subdiabetogenic doses of cortisone acetate upon the blood and urine sugar and on the pancreatic morphology in rabbits.

MATERIAL AND METHODS

The study was carried out on 42 New Zealand white rabbits of either sex weighing between 2.5 and 4.0 kg. which were divided into four groups.

Group I consisted of 12 untreated control animals.

Group II comprised 6 rabbits who each received 1 mg. of crystalline glucagon (Lilly[2]) suspended in corn oil subcutaneously 3 times daily at 10:00 A.M., 4:30 P.M. and 12 midnight. Two animals were sacrificed on the 49th and one each at 63, 72 and 75 days. The remaining rabbit expired on the 75th day.

Group III consisted of 18 rabbits who each were given 1 mg. of crystalline glucagon subcutaneously daily at intervals, as in Group II, together with 1 mg./kg. daily intramuscularly of cortisone-acetate in aqueous suspension (Upjohn[3]). One rabbit each was sacrificed at 5 and 16 days, two at 28 days, four at 46 days, and one each at 53, 66 and 104 days. Seven animals expired at intervals from 4 to 30 days after the start of treatment.

Group IV consisted of 6 rabbits who each received 1 mg./kilo intramuscularly of cortisone acetate daily. One each was sacrificed at 18, 22, 38, 49, 63 and 72 days.

All animals were kept in individual metabolic cages and received Purina rabbit chow and water *ad libitum*. Each animal received 200,000 units of procaine penicillin G and 0.25 gm. of dihydrostreptomycin sulfate (Combiotic-Pfizer) twice weekly. Daily 24-hour urine specimens were collected under toluol. Blood was drawn from the marginal ear vein daily in the morning at approximately 9:30 A.M. and in the afternoon at approximately 1:30 P.M. In addition, in several instances, the blood sugar of an individual animal was followed at ½ hour intervals for 3 to 6 hours. The blood and urinary glucose were determined by the Nelson-Somogyi micromethod (10).

The rabbits were sacrificed by overdosage with barbiturates. The tail of the pancreas, of sacrificed, but not of animals which expired spontaneously, was placed immediately into Zenker-formol (20%) solution for histologic study. After paraffin embedding, slides were stained by the following methods: Iron hematoxylin, phosphotungstic acid hema-

---

[2] Crystalline Glucagon (Lot #258-234B-54-2) was supplied by the Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana, through the courtesy of Dr. W. R. Kirtley.

[3] Cortisone Acetate was supplied by the Upjohn Company, Kalamazoo, Michigan, through the courtesy of Dr. C. J. O'Donovan.

toxylin, modified Masson trichrome (11, 12), modified aldehyde fuchsin-trichrome (13, 14), a modification of Gomori's chrome-alum-hematoxylin (12, 15), the Periodic Acid Leukofuchsin-trichrome (14, 16) controlled by diastase digestion for glycogen identification.

This battery of staining techniques was utilized, in conjunction, in order to characterize more precisely the structural elements of the pancreas and to help avoid any confusion in regard to the definitive identification of the A cells. Some of the methods used, i.e., the iron hematoxylin, phosphotungstic acid hematoxylin and the modified Masson trichrome stains demonstrate A cells by their siderophilia. Others, i.e., the Periodic Acid Schiff-Trichrome, the aldehyde fuchsin and the chrome alum hematoxylin ponceau fuchsin methods show these cells by their acidophilia. It should be noted that, when the chrome alum hematoxylin-phloxin stain is used according to Gomori's original prescription (15), without adequate differentiation by phosphomolybdic acid, pink staining cells may be seen in the islets which are not, but may be mistaken for A cells.

RESULTS

Group I: The untreated control rabbits showed reducing substances in urine which varied from zero up to 0.9 gm. with a mean of 0.52 gm. in 24 hours. Therefore, values above 1.0 gm. per 24 hours were considered significant. There were no significant differences between the morning and afternoon blood sugar values which varied between 93 and 158 mg.% with a mean of 127 mg.% A non-fasting blood sugar value of greater than 160 mg.% was considered a significant elevation.

In the normal pancreas the A cells stood out clearly even with low magnification due to the intense staining of their cytoplasm (Fig. 2); however, not every islet contained these cells. There were no clear cut differences which could be used as criteria for distinguishing the nuclei of the A, B and D cells. All of the small intralobular ductules had a similar histologic appearance and continuity betweeen these structures and islets was only rarely observed.

Group II: The animals treated with glucagon generally maintained their weight. They showed an intermittent hyperglycemia with morning blood sugar values varying between 82 and 175 mg.% and afternoon values ranging from 106 to 405 mg.%. The total 24-hour urinary glucose output varied from 0.2 to 5.8 gm. In general, the severity of the hyperglycemia and glycosuria was greater in the early phases of the experiments and tended to diminish as the duration was extended. The blood and urine sugar values for a representative rabbit are illustrated in Figure 1A.

In the pancreas of these rabbits the A cells seemed to be markedly diminished in number and did not stand out clearly as in the normal (Fig. 3). There were but few well stained A cells present as well as an occasional cell in which only part of the cytoplasm retained the normal tinctorial characteristics of the A cell. Frequent cells were present in the islets which appeared to be poorly stained A cells. These cells showed diffuse cytoplasmic acidophilia and siderophilia which was fainter and duller than that of the normal A cell. Their nuclei were smaller, more chromatic and rounder

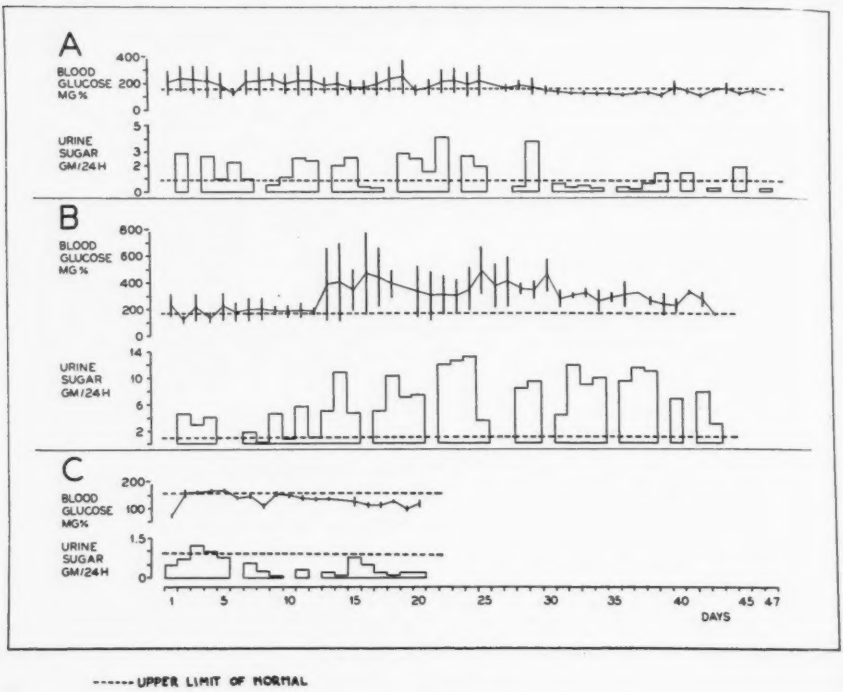
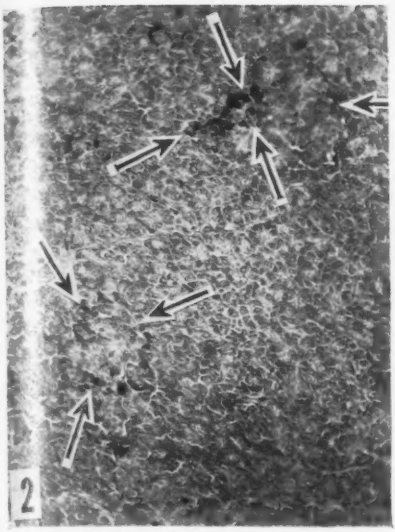


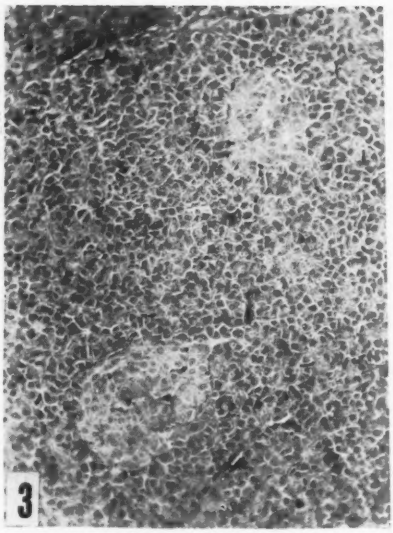
FIG. 1. Daily morning and afternoon blood glucose levels and their average as well as the 24 hour urinary glucose output of three representative rabbits who received respectively: A) 1 mg. of crystalline glucagon in corn oil subcutaneously three times daily. B) Glucagon as in A as well as 1 mg./kg. daily of cortisone acetate intramuscularly. C) Cortisone acetate alone as in B.

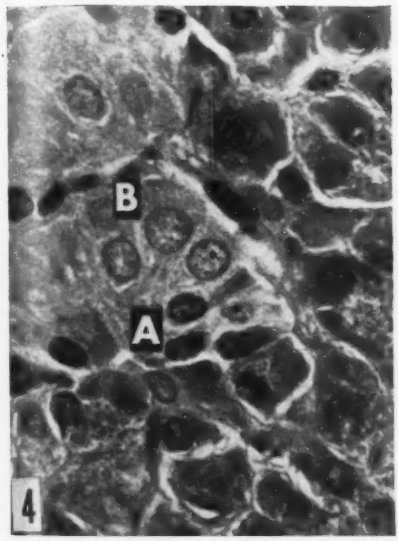
then those of B cells (Fig. 4). Furthermore, nuclei were seen in the islets, similar to those of identifiable A cells, which were either naked or surrounded by ragged faintly staining cytoplasm (Fig. 5). The cytoplasm of these latter was tinctorially quite distinct from the more homogenous cytoplasm of the D cell.

The B cells showed frequently moderate degranulation and the nuclei were generally larger, more ovoid and had more sparsely distributed chromatin than that of identifiable A cells (Fig. 4). Furthermore, the nucleus as well as the entire cell appeared larger than in the normal rabbit. The islet contours were less regular than in the untreated animal.

Group III: The rabbits treated simultaneously with glucagon and cortisone either maintained their weight or showed only a very moderate weight loss of 5 to 10% of their initial weight. In these animals the morning dose of glucagon produced a marked hyperglycemic response which was fairly well sustained until the time for the second dose of glucagon (Fig. 6). The daily blood sugar determinations revealed a severe intermittent hyper-







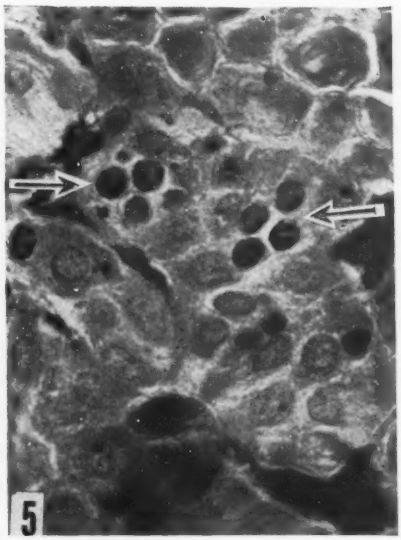


FIG. 2. Pancreas of untreated rabbit showing islet with distinct A cells (arrows). Chrome alum hematoxylin-ponceau-fuchsin stain. ×145.

FIG. 3. Pancreas of rabbit treated for 75 days with glucagon showing islets without apparent A cells. Chrome alum hematoxylin-ponceau-fushsin stain. ×145.

FIG. 4. High power of pancreas of same rabbit as in Fig. 3 showing portion of islet. A cells (A) with poorly stained cytoplasm are present. Their nuclei are smaller and more chromatic than those of adjacent B cells (B). Modified Masson trichrome stain. ×985.

FIG. 5. High power of pancreas of same rabbit as in Figs. 3 and 4 showing part of islet. There is a group of small chromatic nuclei (arrows) with poorly recognizable cytoplasm, thought to represent degranulated A cells. The nuclei of adjacent B cells are much larger. Phosphotungstic acid hematoxylin stain. ×985.

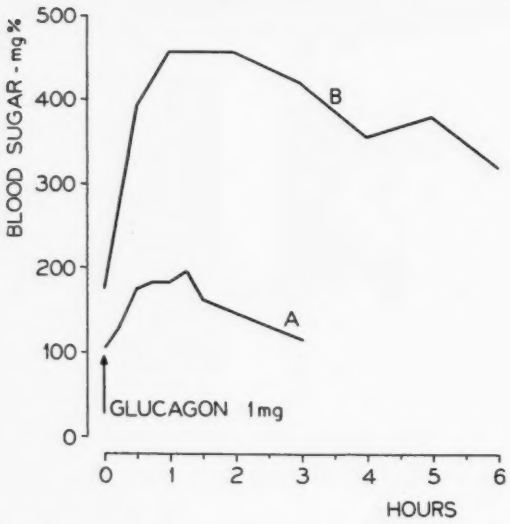
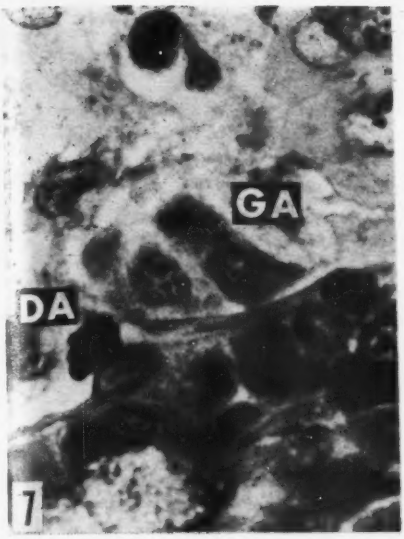


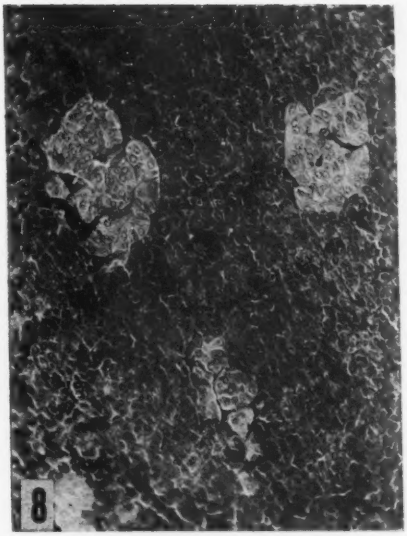
FIG. 6. Comparison of blood sugar response to a single subcutaneous injection of 1 mg. of crystalline glucagon in corn oil of (A) an untreated rabbit and of (B) a rabbit which also had been receiving 1 mg./kg. daily of cortisone acetate intramuscularly.

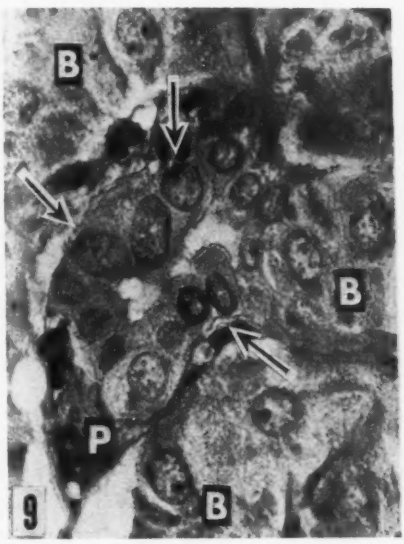
glycemia. The morning values were frequently within normal limits during the early stages of treatment. Thereafter, for a short time they tended to rise reaching as high as 440 mg.%. The afternoon blood sugar levels were elevated from the beginning. This elevation became extreme as treatment continued with occasional values as high as 840 mg.%. After 6 weeks the morning and afternoon glycemic levels were frequently less than prior to that time. The urinary glucose output was markedly elevated reaching as much as 13 gm. per 24 hours and in general varied concomitantly with the blood sugar levels. The daily blood sugar values as well as the 24 hour glucose output of a representative animal are seen in Figure 1B.

Morphologically, the A cells showed tinctorial changes similar to those observed in rabbits treated with glucagon alone. Although there was a suggestive alteration of A cells in the pancreas of the rabbits sacrificed after 16 days, this alteration became obvious in most instances after 28 days, and was most pronounced after 53 to 104 days. Figure 7 demonstrates a partly degranulated A cell from one of these animals. In 2 rabbits which were similarly treated for 46 days each no obvious changes in A cells were seen.

In those animals treated over prolonged periods of time the islets were hyperplastic and had irregular contours (Fig. 8). The B cells were frequently degranulated and larger than normal. The nuclei were also increased in size thus accentuating the contrast with the A cell nuclei. Hyperplastic ductular epithelium was seen contiguous with or within islets







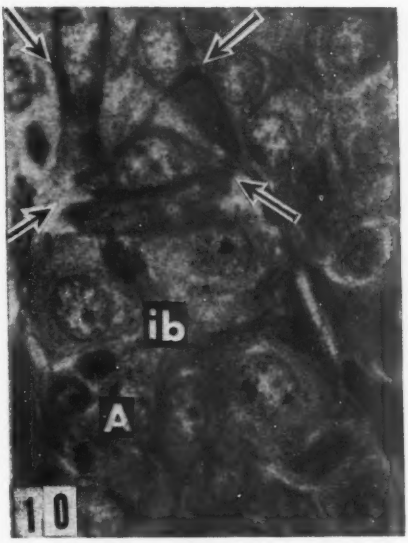
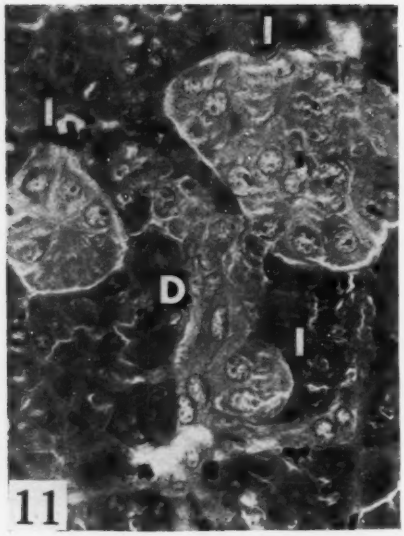


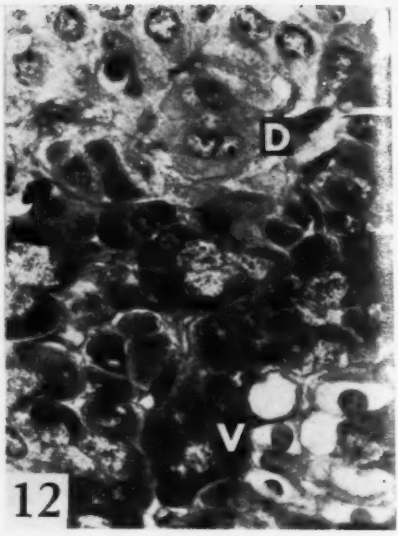
FIG. 7. Pancreas of rabbit treated for 51 days with glucagon and cortisone showing a granulated (GA) as well as partly degranulated (DA) A cell. Modified Masson trichrome stain. ×1535.

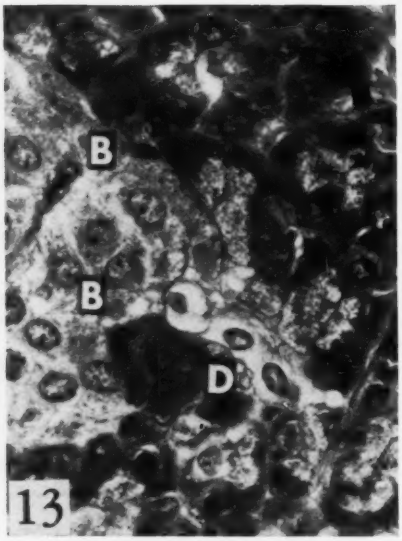
FIG. 8. Pancreas of rabbit treated for 104 days with glucagon and cortisone showing considerable islet hyperplasia and no apparent A cells. Modified Masson trichrome stain. ×145.

FIG. 9. Pancreas of same rabbit as in Fig. 8, showing intraislet proliferating hyperplastic ductules (arrows) with a primitive B cell (P) originating from it. The adjacent B cells (B) are more mature. Modified Masson trichrome stain. ×985.

FIG. 10. Pancreas of same rabbit as in Figs. 8 and 9, showing large immature B cells (ib) with occasional sparse intracytoplasmic aldehyde fuchsin positive granules. Adjacent mature but degranulated B cells (B) are delineated by heavily stained aldehyde fuchsin positive borders (arrows). There are occasional more chromatic nuclei (A) with indistinct cytoplasm probably representing degranulated A cells. Modified aldehyde fuchsin trichrome stain. ×1230.







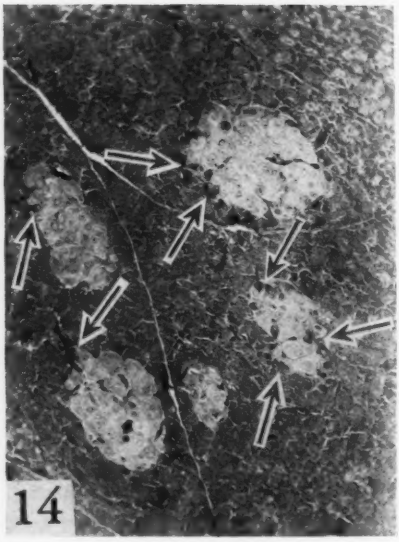


FIG. 11. Pancreas of same rabbit as in Figs. 8, 9 and 10, showing hyperplastic ductule (D) with apparently newly formed islets (I) composed only of B cells. Modified Masson trichrome stain. ×585.

FIG. 12. Lower power view from same field as in Fig. 7, showing proliferating non-vacuolated ductules (D) within an islet and an adjacent vacuolated ductule (V). Also seen are two A cells one of which is partly degranulated (see Fig. 7). Modified Masson trichrome stain. ×735.

FIG. 13. Pancreas of same rabbit as in Figs. 7 and 12. A markedly vacuolated ductule (D) is in direct continuity with an islet showing but moderately vacuolated B cells (B). Modified Masson trichrome stain. ×290.

FIG. 14. Pancreas of rabbit treated for 38 days with cortisone alone showing distinct A cells (arrows). There is moderate islet hyperplasia and some B cell degranulation. Chrome alum hematoxylin-ponceau-fuchsin stain. ×145.

and new formation of B cells from duct epithelium could be traced. This was evidenced by intrainsular young B cells which were in direct contiguity with duct epithelium and with surrounding more mature B cells (Fig. 9). The immature cells were large with vesicular nuclei, and sparsely distributed aldehyde-fuchsin positive granules in the cytoplasm (Fig. 10). Adjacent older B cells were smaller, frequently degranulated and showed aldehyde-fuchsin positive cell borders (Fig. 10). Occasionally, small young islets were observed which in an appendage-like fashion were loosely attached to a duct (Fig. 11). There were also in some instances isolated granulated B cells seen within cords of degranulated older B cells with aldehyde-fuchsin positive borders. These isolated granulated B cells are thought to represent a new generation as compared with the older B cells which have become degranulated under the influence of hormonal treatment.

In severely diabetic rabbits there was marked glycogen infiltration (vacuolization) of ductular epithelium and of many of the B cells. However, it was noteworthy that the ductules from which new B cells appeared to form were frequently hyperplastic but devoid of glycogen infiltration (vacuolization) (Fig. 12). Similarly the cells identified as young B cells did not contain glycogen even when present in other B cells. On the other hand, pancreatic islets were also frequently continuous with or even traversed by vacuolated ductules (Fig. 13). As previously reported in animals treated with larger amounts of adrenal steroids (17, 18, 19, 20) the present animals similarly showed intraislet dilatation of ductules and occasional pancreatic lobules which consisted of proliferating dilated ductules and dedifferentiated acinar tissue with comparatively intact islets.

Group IV: In the cortisone treated rabbit only a very moderate weight loss similar to that of Group III was observed. There were no differences between the morning and afternoon blood sugar values which varied from 73 to 170 mg.%. The 24 hour urine glucose output varied from zero to 1.2 gm. The blood sugar values and urinary glucose output of a representative rabbit are seen in Figure 1C. In these pancreas' the A cells stood as clearly as in the normal rabbit (Fig. 14). There was some B cell degranulation, very mild degrees of glycogen infiltration and some ductular and islet hyperplasia. The islet hyperplasia appeared to be similar in extent to that observed in animals treated for equal periods of time with cortisone and glucagon but was greater than that seen with glucagon alone. There was also some intraislet ductular dilatation and alteration of lobules as described for Group III.

DISCUSSION

The prolonged administration in rabbits of glucagon or cortisone alone produces only a mild hyperglycemia. On the other hand, when glucagon is given in conjunction with cortisone, severe hyperglycemia and glycosuria

is observed. This finding is in accord with our previous demonstration in acute experiments in rabbits of the potentiating action of cortisone on glucagon hyperglycemia (7, 8). It is also in agreement with the reports of others (9) that adrenocorticotropic hormone and cortisone exaggerated glucagon induced hyperglycemia in rats.

Morphologically, the most striking observations were the changes in the A cells which were of corresponding extent in both groups of glucagon treated rabbits. Although identifiable A cells were generally found in these pancreas, they seemed to be diminished in number and frequently showed faintly staining cytoplasm or were partly degranulated (Figs. 3, 4, 7). Moreover, the nuclei appeared to be smaller and more chromatic than in the normal. Groups of similar nuclei with indistinct cytoplasm were considered to represent completely degranulated A cells (Fig. 5). The most pronounced changes were seen in the animals treated for the longest periods.

To our knowledge there is only one morphologic study reported (1, 2) in which atrophy of A cells was found in rabbits and rats after glucagon administration. This atrophy was deduced, mostly, from a decrease in the size of the A cell nuclei. Other workers (6) did not mention pancreatic A cell changes after 7 days of administration of glucagon in corn oil to rats and dogs.

The present observations, of the effect of glucagon on the A cells, suggest that this is an example of suppression of endogenous secretion of a hormone by its exogenous administration, similar to that seen with many hormones. This supports the viewpoint that glucagon is a hormone and that it is produced by the A cells (3, 4, 5). It is in opposition to the previous conclusion derived from our studies with cobaltous chloride that the A cells are not the source of glucagon (21). This latter conclusion was based, partly, on the fact that there was no reduction in the hyperglycemic action of pancreatic extracts from cobalt-treated dog pancreas as compared to the normal (22, 23). It was stated, at that time, that since many A cells were still left in the pancreas after cobalt treatment and since the method of glucagon assay was qualitative rather than quantitative, a moderate reduction in glucagon content may not have become apparent. It has since been found that with this method it is only when the A cells are completely or almost completely absent from the pancreas that there is a diminished hyperglycemic action of extracts (24, 25).

Other morphologic alterations of the pancreas seen in the present experiments are comparable to those reported in almost all other types of hormonally induced experimental hyperglycemia (26, 27). These changes included B cell degranulation and glycogen infiltration of duct epithelium and of B cells. They were most marked in animals treated conjointly with cortisone and glucagon, suggesting that hyperglycemia is the responsible factor. Furthermore, the degree of glycogen infiltration of ducts and of B

cells corresponded with the duration and severity of the diabetic state. In mild diabetes, mainly ductular epithelium and only occasional B cells were affected. In more severe diabetes, the B cell vacuolization also became pronounced. This is in accord with the results of previous work in which it was shown that ductular glycogen deposition will occur prior to that in B cells (17, 19, 27, 28, 29). These findings have led to the conclusion that B cell glycogen deposition is not a result of functional exhaustion of these cells, but that it is only one aspect of the increased intracellular glycogen occurring in diabetes.

B cell destruction with the development of metadiabetes has been shown to result after prolonged hyperglycemia in dogs and cats (30, 31, 32, 33) but not in rodents (17, 19, 20, 34, 35). Similarly in the present study despite a longer lasting and more severe hyperglycemia induced by cortisone and glucagon than ever previously established in rabbits by hormonal treatment, there was no evidence of B cell destruction. On the contrary, as treatment was prolonged, there was an apparent diminution in the diabetogenic effectiveness of the hormones. The question might be raised as to whether this is due to the suppression of endogenous glucagon production or to increased insulinogenic activity in the pancreas. The former hypothesis seems to be invalidated by the fact that the amount of glucagon administered is in marked excess and that a similar amelioration is seen in diabetes induced by other hormones (17, 36, 37). It, therefore, seems more logical to assume that, this resistance is a result of increased insulinogenesis. This idea is supported by the morphologic findings in the pancreas which included B cell degranulation, hyperplasia of the islets and neogenesis of B cells from duct epithelium.

In the present study the earliest stage in new B cell formation was the presence within hyperplastic ductules of single cells which were larger and differed tinctorially from the adjacent epithelial cells. These cells were recognized as primitive B cells because of their similarity to young intraislet B cells, which contained a few scattered aldehyde-fuchsin positive intracytoplasmic granules. Furthermore, these cells were seen in direct continuity with definitely recognizable mature B cells (Figs. 9 and 10).

In the embryo it has been claimed that the B cells may derive either by differentiation from primitive ductular epithelium, or by the transformation of acinar cells (38, 39). Under various conditions in post fetal life new formation of B cells has been claimed to occur from acinar cells and from other islet cell types as well as from duct epithelium (40–45). The main evidence advanced for the former types of transformation has been either geographic contiguity or the supposed presence of different types of intracellular granules in the same cell (40, 42, 43). However, it is generally acknowledged that although primitive cell types may differentiate in various directions, the mature cell only reproduces itself. In our studies no evidence of B cell neogenesis from acinar cells, A cells or D cells was found. This is in

agreement with the observations of others that apparent acino-insular transformation or the interchangeability of various islet cell types is an artefact either due to superimposition of two different cell types or to a confusion of mitochondria with intracytoplasmic secretory granules (39).

An additional finding which strongly supports these conclusions is the observation of the presence in the pancreas of the severely diabetic rabbit of two different categories of small intralobular ductules. One type showed marked vacuolization due to glycogen infiltration while the other type was hyperplastic and non-vacuolated. It is from ductules of this latter type which are thought to correspond with the primitive cell cords described in the adult guinea pig (46) that B cell neogenesis was observed to occur.

Acknowledgments

The authors wish to acknowledge the technical assistance of Miss Josephine McLeod. We are grateful to Mrs. Renee Nakrinsky for editing and typing the manuscript. The photographs were prepared by Mr. Herbert A. Fischler.

REFERENCES

1. KRACHT, J.: *Naturwissensch.* **41:** 336. 1954.
2. KRACHT, J.: *Naturwissensch.* **42:** 50–51. 1955.
3. CAVALLERO, C.: *Rev. Can. de Biol.* **12:** 509–529. 1953.
4. FOA, P. P.: Glucagon, the Hyperglycemic-glycogenolytic Hormone of the Pancreas in: Advances in Internal Medicine, Vol. VI, 1954, The Year Book Publishers, Inc., pp. 29–58.
5. KORP, P. AND P. M. LE COMPTE: *Diabetes* **4:** 347–366. 1955.
6. SALTER, J. M., I. W. F. DAVIDSON AND C. H. BEST: *Diabetes* **6:** 248–252. 1957.
7. VOLK, B. W., S. S. LAZARUS AND H. LEW: *Metabolism* **4:** 503–512. 1955.
8. LAZARUS, S. S., B. W. VOLK AND H. LEW: *J. Clin. Endocrinol. & Metab.* **17:** 542–551. 1957.
9. CAVALLERO, C. AND B. MALANDRA: *Acta Endocrinologica.* **13:** 79–88. 1953.
10. NELSON, N.: *J. Biol. Chem.* **153:** 375–380. 1944.
11. MASSON, P.: *J. Tech. Methods.* **12:** 75–94. 1929.
12. BENCOSME, S. A.: *A.M.A. Arch. Path.* **53:** 87–97. 1952.
13. GOMORI, G.: *Am. J. Clin. Path.* **20:** 665–666. 1950.
14. LAZARUS, S. S.: Unpublished.
15. GOMORI, G.: *Am. J. Path.* **17:** 395–406. 1941.
16. LILLIE, R. D.: Histopathologic Technique and Practical Histochemistry, The Blakiston Co., Inc., N. Y. 1954.
17. LAZARUS, S. S. AND S. A. BENCOSME: *Proc. Soc. Exp. Biol. & Med.* **89:** 114–118. 1955.
18. BENCOSME, S. A. AND S. S. LAZARUS: *Arch. Path.* **62:** 285–295. 1956.
19. LAZARUS, S. S. AND S. A. BENCOSME: *Am. J. Clin. Path.* **26:** 1146–1156. 1956.
20. VOLK, B. W. AND S. S. LAZARUS: *Am. J. Path.* **34:** 121–136. 1958.
21. VOLK, B. W., S. S. LAZARUS AND M. G. GOLDNER: *A.M.A. Arch. Int. Med.* **93:** 87–106. 1954.
22. GOLDNER, M. G., B. W. VOLK AND S. S. LAZARUS: *J. Clin. Endocrinol. & Metab.* **14:** 184–192. 1954.
23. LAZARUS, S. S., B. W. VOLK AND M. G. GOLDNER: *Metabolism.* **3:** 449–455. 1954.

25. Bencosme, S. A., E. Liepa and S. S. Lazarus: *Proc. Soc. Exp. Biol. & Med.* **90:** 387–392. 1955.
26. Bencosme, S. A., S. S. Lazarus, E. Liepa, J. Frei and S. Mariz: *Am. J. Path.* **32:** 630. 1956.
27. Warren, S. and P. M. Le Compte: The Pathology of Diabetes Mellitus, Lea & Febiger, Philadelphia, 1952.
28. Lazarus, S. S. and B. W. Volk: *A.M.A. Arch. Path.* In Press.
29. Lazarus, S. S. and B. W. Volk: *Proc. Soc. Exp. Biol. & Med.* **94:** 610–613. 1957.
30. Lazarus, S. S. and B. W. Volk: *Diabetes* **7:** 15–20. 1958.
31. Allen, F. M.: *J. Metab. Res.* **1:** 5–41. 1922.
32. Richardson, K. C. and F. G. Young: *Lancet* **1:** 1098–1101. 1938.
33. Ham, A. W. and R. E. Haist: *Am. J. Path.* **17:** 787–812. 1941.
34. Lukens, F. D. W. and F. C. Dohan: *Endocrinology* **30:** 175–202. 1942.
35. Hausberger, F. X. and A. J. Ramsay: *Endocrinology* **53:** 423–435. 1953.
36. Franckson, J. R. M., W. Gepts, P. A. Basteine, V. Conrad, W. Cordier and L. Kovacs: *Acta Endocrin.* **14:** 153–167. 1953.
37. Young, F. G.: *Lancet* **2:** 372–374. 1937.
38. Young, F. G.: *New England J. Med.* **221:** 635–646. 1939.
39. Van Campenhout, T.: *Arch. Biol.* **37:** 9–171. 1927.
40. Bencosme, S. A.: *Am. J. Anat.* **96:** 103–152. 1955.
41. Baker, B. L.: A Comparison of the Histologic Changes Induced by Experimental Hyperadrenocorticism and Inanition; In: *Recent Progress in Hormone Research,* Pincus, G. (Ed). Acad. Press, N. Y. **7:** 331–337. 1957.
42. Simard, L. C.: *Rev. Can. de Biol.* **1:** 2–49. 1942.
43. House, E. L.: *Endocrinology* **62:** 189–200. 1958.
44. Gomori, G.: *Arch. Path.* **36:** 217–232. 1943.
45. Florentin, P. and D. Picard: *Rev. Franc. d'endocrin.* **14:** 1–27. 1936.
46. Woerner, C. A.: *Anat. Rec.* **71:** 33–57. 1938.
47. Bensley, R. R.: *Am. J. Anat.* **12:** 297–388. 1911.

THE BIOASSAY OF THYROTROPIN IN SERUM[1]

J. M. McKENZIE

Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston, Massachusetts

ABSTRACT

A bioassay technique for thyrotropin in serum was developed from that of Adams and Purves. Mice were injected with I^{131} and then had endogenous thyrotropin suppressed by the administration of thyroxine followed by thyroid powder. The effect of an intravenous injection of thyrotropin or the test substance was measured by the percentage increase in blood I^{131}. This had a linear relationship to the logarithm of the dose. Sensitivity of 0.025 mu., with an injection volume of 0.5 ml., and an average index of precision (λ) of 0.24 were achieved.

With this technique thyrotropin in the sera of 6 patients with myxedema was measured. Endogenous thyrotropic activity was shown to travel on electrophoresis with gamma globulin of such sera and was also present only in the gamma globulin fraction (II) of Cohn fractions of human plasma.

IT IS 50 years since Rénon and Delille (1) described how the thyroid of rabbits which had been injected with an hypophyseal extract was "le siège de modifications très remarquables." Since the first quantitative assay of thyrotropin in 1932 (2), there have been many others described and many clinical studies reported where the concentration of thyrotropin in human blood or urine has been studied. A bioassay, however, is still to be described which measures unequivocally normal or subnormal concentrations of this hormone in human blood.

Few attempts appear to have been made to concentrate the thyrotropic activity in blood. Fellinger's method (3) has been used by several investigators (4, 5, 6, 7). Albert (8), however, cast considerable doubt on the efficiency of this method, pointing to the poor results obtained when pituitary thyrotropin is added to and recovered from whole blood. This loss was earlier demonstrated by Collard and his collaborators (5). Purves and Griesbach (9) suggested there were dangers of modifying the activity of the circulating thyrotropin during the process of concentration and preferred to use whole serum.

A sensitive assay has been developed, adapted from that of Adams and Purves (10) and the possibility of concentrating serum thyrotropic activity by electrophoresis examined. Preliminary reports on this work have already been published (11, 12).

Received April 22, 1958.

[1] This work formed part of a thesis submitted for the degree of M.D. of the University of St. Andrews, Scotland.

METHODS

Female albino mice, Swiss-Webster strain, obtained from Taconic Farms, New York, were used exclusively. They arrived with a mean weight of 15 gm. and, unless otherwise stated, were fed on finely ground dog biscuit (Ken-l-ration) which was of low iodine content (250 μg./kg.). When blood specimens were required, they were taken by venepuncture after the animals had been kept at a temperature of 29–31° C for 45 minutes. Tail veins were punctured with a 24 gauge needle and the blood which welled on to the skin surface was aspirated by the use of a 1 ml. tuberculin syringe. The tail veins were also used for intravenous injections. Carrier-free radioiodine (I^{131}) was injected intraperitoneally and thyroxine subcutaneously in the dorsal neck region. Thyroxine solutions were made fresh before use, as were the solutions of the thyrotropin standard (U.S.P. Thyrotropin Reference Standard).

In vivo and *in vitro* counting of radioactivity in mouse glands was carried out by means of a suitably shielded scintillation counter. Sufficient total counts were made to give a probable error (P.E.) of less than 1%. I^{131} in blood was counted by plating 0.1 ml. specimens on aluminum planchettes. When the blood was dry, radioactivity was counted by either a gas flow counter or a gas flow end-window counter (Nuclear-Chicago Model D-47). For counting done with the former instrument P.E. averaged 3% and with the latter, which was used in conjunction with an automatic sample changer, 2%.

Blood to be assayed was obtained from various patients by venepuncture and refrigerated overnight before the serum was separated. Serum was stored at 4° C until shortly before assay.

Starch block electrophoresis was carried out by the method described by Kunkel and Slater (13), with a few modifications. An almost dry suspension was made of potato starch in borate buffer, pH 8.6. The potato starch was previously washed with this buffer until the washings were similarly alkaline. A barely fluid suspension of starch in serum and buffer was poured into a slit in the center of the starch block. Three ml. specimens of serum were fractionated, the electrophoresis being carried out at 4° C for various periods ranging from 6 to 15 hours, with a current of 2–10 milliamps. After electrophoresis the block was cut into 1 cm. segments and eluted with 1% saline. The distribution of protein was determined by spectrophotometry at 278 mμ. and appropriate eluates combined, dialyzed against tap water and finally lyophilized.

The resulting powders were weighed and dissolved in 1% saline, with minimal alkali where necessary, immediately before assay. Their identity was subsequently confirmed by electrophoresis on paper at pH 8.6, whole serum being simultaneously electrophoresed.

RESULTS

A. *Development of the assay technique*

1) *Suppression of endogenous thyrotropin.* The effect of exogenous thyroid hormone on the suppression of endogenous thyrotropin secretion was investigated by measuring the effect on radioiodine uptake (Figs. 1 and 2). A single subcutaneous injection of 10 μg. *l*-thyroxine increasingly suppressed the uptake for 4 days. There was rapid return to normal (or supranormal) uptake figures in 5th and 6th days. Thyroid powder (U.S.P.) added to the "Ken-l-ration" diet at 0.066% concentration gave optimal suppression of uptake. The routine adopted therefore was to give a single subcutaneous injection of 10 μg. thyroxine and add 0.066% thyroid powder to the diet at this point.

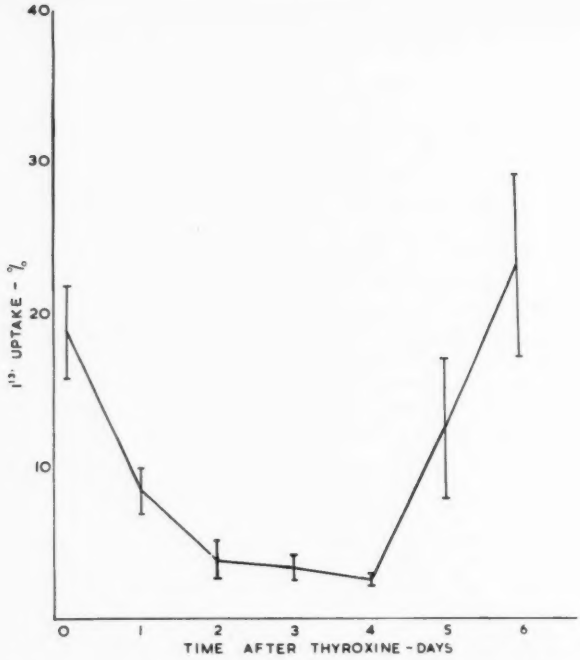


FIG. 1. Effect of a single injection of *l*-thyroxine on I^{131} uptake. 10 μg. was injected subcutaneously into each animal. Mean and standard error of the mean of 4 observations are shown in each instance.

2) *Maximum permissible thyroid content of* I^{131}. Thyrotropic effect was to be measured by the increase in circulating radioactivity resulting from an intravenous injection of the test substance. It was desirable, therefore, to have the maximum quantity of I^{131} in the thyroid gland of the mice, short of producing radiation damage. This quantity was estimated as shown in Fig. 3. Mice were injected with various doses of I^{131} and *in vivo*

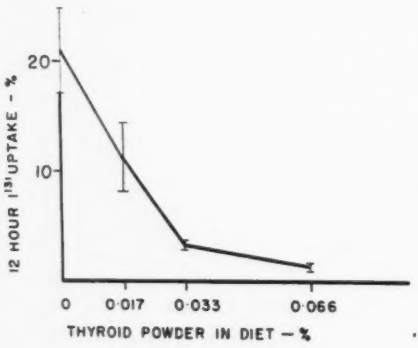


FIG. 2. Effect on I^{131} uptake of feeding thyroid powder for one week. Mean and standard error of the mean of 5 observations are shown in each instance.

neck counts carried out at 12 hours and frequently over the next 17 days. The release of I^{131} from the mouse glands was plotted. It was assumed that radiation damage was associated with an increased rate of release from the animals' thyroids, i.e., an increase in steepness of the release slope. Thus it was determined that more than 2 μc. I^{131} in the thyroid gland of the mouse might be associated with radiation damage. Since after a week on the low iodine diet I^{131} uptake averaged 20%, routinely 8 μc. I^{131} was injected, thus achieving an average thyroid gland content of 1.6 μc.

Thyroxine injected subcutaneously immediately following the I^{131} was found not significantly to affect the uptake. The routine for preparing mice for assay purposes is therefore as shown in Table 1. Three days were allowed for the clearance of blood radioiodide. This was the procedure adopted by Adams and Purves (10) in their assay technique and it proved to be appropriate for this method also.

TABLE 1. PREPARATION OF MICE FOR ASSAY PURPOSES

Day	Procedure
1– 8	Low iodine diet
8	Intraperitoneal injection of 8 μc. I^{131} followed by the subcutaneous injection of 10 μg. *l*-thyroxine
	Addition to diet of 0.066% thyroid powder
9–11	Time allowed for uptake or excretion of radio-iodide
12–14	Animals available for assays

3) *Measurement of thyrotropic activity.* Thyrotropin was injected intravenously. The time of maximal effect on circulating radioactivity was found to occur after 2 hours (Fig. 4).

Comparison was made of the dose-response relationship in 4 ways. The increase in blood I^{131} at 2 hours was calculated both as a % increase on the initial activity and as an absolute increase. The latter was expressed as counts per minute per 0.1 ml. of blood. Both of these effect metameters were plotted against the dose of thyrotropin expressed either on a logarithmic or arithmetic scale. Absolute increase with log dose, and % increase with arithmetic dose, were found not to have a linear relationship. Absolute increase with arithmetic dose and % increase with log dose both gave linear results. Statistics applicable to the latter relationship are well recognized so it was adopted for assay purposes. A typical assay curve is shown in Figure 5.

4) *Specificity.* Various substances were used to determine the specificity of the technique. Injection of 0.2 ml. saline was made on 55 occasions and the effect judged after 2–3 hours. A slight fall in radioactivity occurred, with a mean response of 97% (i.e., % of an initial blood radioactivity) and a standard deviation of 4%.

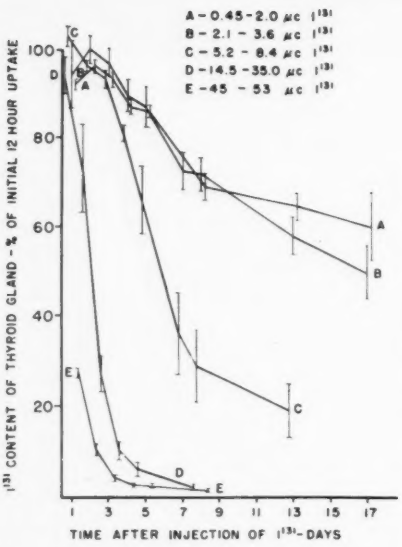

A - 0.45 - 2.0 μc I131
B - 2.1 - 3.6 μc I131
C - 5.2 - 8.4 μc I131
D - 14.5 - 35.0 μc I131
E - 45 - 53 μc I131
I131 CONTENT OF THYROID GLAND - % OF INITIAL 12 HOUR UPTAKE
100
80
60
40
20
A
B
C
D
E
1 3 5 7 9 11 13 15 17
TIME AFTER INJECTION OF I131 - DAYS

FIG. 3. Estimation of maximum permissible I^{131} content of thyroid gland. Mean and standard error of the mean are shown in every instance.

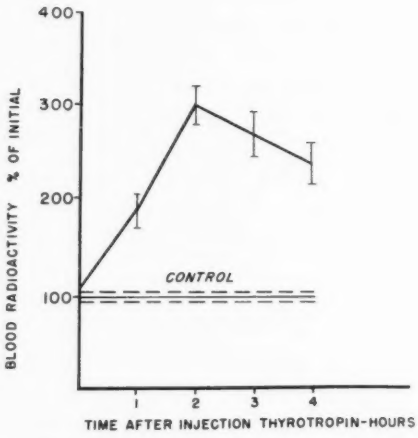


FIG. 4. Time of maximum effect of I.V. thyrotropin on blood I^{131}. Mean and standard error of the mean of results in 6 mice are shown in each instance. Control is the mean and standard deviation of results from the injection of saline.

Since the handling of the mice was obviously stressful to them, the effect of adrenaline and of hydrocortisone was studied. Adrenaline in doses of 0.05 μg. and 1 μg. per animal was injected intravenously and the effect on the blood radioactivity determined after 2 hours. The results were not significantly different from those obtained with the injection of saline. One μg. was little short of the lethal dose of adrenaline by the intravenous route. (On a weight basis it would be equivalent to about 3.5 mg. in a 70 kg. man.)

Similarly, doses of 0.01 mg. and 0.1 mg. of hydrocortisone produced no significant elevation of blood radioactivity 2 hours after their intravenous injection. 0.1 mg. hydrocortisone is comparable, again on a weight for weight basis, with about 350 mg. in man.

As is detailed later, various extracts of serum and plasma produced no thyrotropin-like effect. A glacial acetic acid extract of human pituitaries was also tested.[2] This was a highly potent preparation of growth hormone and by other standards contained very little thyrotropin. It had a protein content of 25 mg. per ml. Doses of 0.25 ml. of this preparation demonstrated a small thyrotropin-like effect, quite acceptable as the result of minimal contamination of the extract with thyrotropin.

[2] I am grateful to Dr. M. S. Raben for the supply of this extract.

5) *Sensitivity and precision.* 0.025 mu. was the lower limit of detection of thyrotropin by this technique. This was with a volume of injection of 0.2 to 0.5 ml. 1.6 mu. was the maximum dose tested and this was still within the range of linear responsiveness.

The average index of precision (λ) was 0.24. Table 2 lists mean standard deviations, slopes and values for λ for 11 assays with 3 doses of standard thyrotropin and similar results calculated from assays with 2 doses of standard.

TABLE 2. PRECISION OF ASSAY

No. of doses	No. of assays	s mean	b mean	λ (s/b)		
				Mean	S.D.	Range
3	11	57.9	270.2	0.236	0.074	0.137–0.345
2	11	60.6	267	0.243	0.066	0.128–0.378
2–3	22	59.3	268.6	0.240	0.070	0.128–0.378

Doses of thyrotropin ranged from 0.025 to 1.6 mu. s, b and λ were calculated as described by Gaddum (14).

From this index the approximate minimum error to be expected when standard and unknown have equal average effects may be calculated (14). The minimum variance $[V(M)]$ of the logarithm of the result of the assay equals $4\lambda^2/N$, where N denotes the total number of animals used in an assay. It was found convenient to use a 4-point assay technique with 6 observations for each of 2 doses of the standard thyrotropin and the unknown preparation. Thus with a total of 24 mice:

$$V(M) = 4\lambda^2/N = 4 \times (0.24)^2/24 = 0.0096$$

$\therefore$ Minimum standard deviation of the logarithm of the result

$$= \sqrt{0.0096} = 0.098$$

Antilog 0.098 = 1.253

The error of an assay would therefore be about 25%.

B. *Application of the assay method*

Sera of patients with myxedema. Thyrotropin was detected in the whole serum of one patient with acromegaly which was no longer progressive and of 6 with myxedema. The diagnosis of myxedema was confirmed by estimation of serum protein-bound iodine in all cases and in 5 also by measurement of the thyroid gland uptake of I^{131}.[3] Results of the assays in 4 of these patients with myxedema are shown in Table 3. The statistical calculation of these was as described by Gaddum (14).

[3] Dr. C. E. Cassidy kindly gave me clinical data on these patients who were under his care.

TABLE 3. THYROTROPIN CONCENTRATION IN SERA FROM PATIENTS WITH MYXEDEMA

Patient No.	Assay design (U:S)*	Estimate of thyrotropin per ml. serum (P=0.95)		
		Mean	Upper limit	Lower limit
3	1:2	0.64 mu.	1.06 mu.	0.03 mu.
5	1:2	0.12 mu.	0.28 mu.	0.06 mu.
6	1:3	0.13 mu.	0.21 mu.	0.08 mu.
7	1:2	0.343 mu.	0.332 mu.	0.358 mu.

* U = unknown, i.e. serum.
S = standard thyrotropin.
Calculation of the fiducial limits was carried out as described by Gaddum (14).

Fractionation of sera from myxedematous patients. Sera from 5 patients with myedema were fractionated by the technique of starch block electrophoresis. Separate fractions containing albumin, alpha globulins, beta globulins and gamma globulins were obtained in each case and assayed for thyrotropin. This activity was found on each occasion to be with the gamma globulin fraction only. Detailed results of 3 such assays are given in Table 4.

Assay of Cohn plasma fractions. Cohn fractions of human plasma (obtained from the American Red Cross) were tested. Two per cent solutions

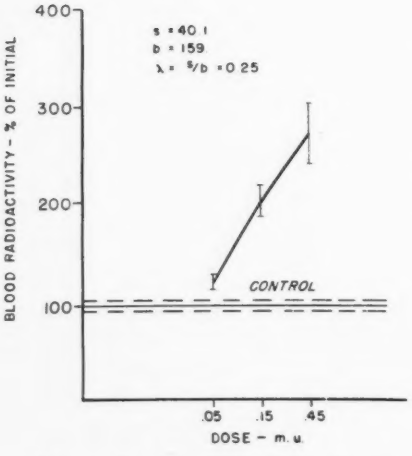


FIG. 5. Dose-response curve. Dose is plotted on a logarithmic scale. Mean and standard error of the mean are shown in each instance. s = mean standard deviation. b = slope.

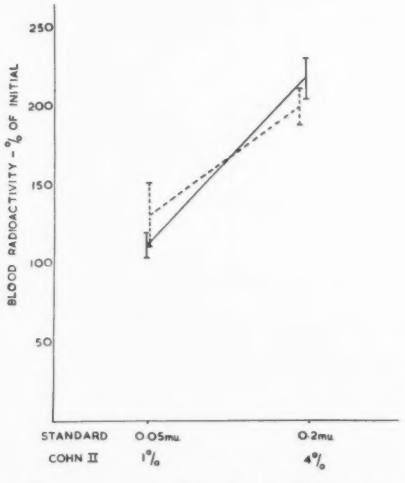


FIG. 6. Assay of Cohn fraction II. Results obtained with U.S.P. Thyrotropin Reference Standard are plotted in solid lines and with Cohn fraction II in interrupted lines. Mean and standard error of the mean of 6 observations are shown in each instance. Each injection had a volume of 0.2 ml.

TABLE 4. ASSAY OF SERUM γ GLOBULIN FRACTIONS FROM PATIENTS WITH MYXEDEMA

Patient No.	Assay design (U:S)*	Estimate of thyrotropin per ml. serum (P = 0.95)		
		Mean	Upper limit	Lower limit
5	2:2	0.70 mu.	1.36 mu.	0.43 mu.
6	1:2	0.15 mu.	0.30 mu.	0.07 mu.
8	1:3	0.362 mu.	0.62 mu.	0.21 mu.

* U = unknown, i.e. serum.
S = standard thyrotropin.
Calculation of the fiducial limits was carried out as described by Gaddum (14).

of fractions I, IV-1 and IV-4 and a 5% solution of fraction V (all lacking gamma globulin) were found to have no demonstrable thyrotropic activity. Fraction III (mainly beta globulin with a small content of gamma globulin) had minimal demonstrable activity, in a 2.7% solution. Fraction II (gamma globulin with a small content of beta globulin) produced definite responses and was tested at concentrations of 1% and 4% as a 4-point assay (Fig. 6). Thyrotropin content was found to be 0.166 mu. per ml. plasma (assuming fraction II to be 0.66% in normal plasma) (15) with a 95% probability range of 0.1–0.28 mu. The parallelism of the response to the Cohn fraction with the response to the thyrotropin standard was tested as described by Gaddum (14). Deviation from parallel was not significant (P = 0.2–0.1).

DISCUSSION

According to Albert (8) in 1947 it could not be stated that "TSH has been unequivocally found in serum." Since then a few reports have been published of apparent unequivocal demonstration of the presence of thyrotropin in the serum of humans with various states of thyroid function (9, 16, 17, 18). The sensitivity of the assay techniques in the last three of these reports was 0.1 mu. (19), 0.15 mu. (20) and 0.1 mu. (10). The sensitivity of the method described here is superior to these and its success in detecting thyrotropin in serum from patients with myxedema was not unexpected. With the facility of injecting 0.5 ml. of serum into the test animal, a sensitivity of 0.025 mu. thyrotropin should make the technique applicable in a variety of clinical states.

The average concentration in normal human serum is not yet established. Gilliland and Strudwick (17) measured thyrotropin in the serum of 2 normal individuals and in the pooled serum of 6 volunteers. Their average for these 3 tests was 39 μg. thyrotropin per 100 ml. This was equivalent to 0.165 mu. thyrotropin/ml.[4] However, they failed to detect activity in

[4] The potency of the standard preparation (R 377158) used by Gilliland and Strudwick was 0.433 U.S.P. unit/mg. I am grateful to D. Purdie of The Armour Laboratories, Eastbourne, Sussex, England, for this information.

the sera of another 2 normal subjects. DiGeorge, D'Angelo and Paschkis (21) reported 0.4 mu./ml. as the average normal in their experience. In the assay of Cohn fractions reported here, virtually all the thyrotropic activity was found with fraction II. If this were accepted as the total activity, an estimate of 0.166 mu./ml. is obtained. Despite the rationalization of Greer and Shull (22) that there must be in normal humans less than 0.06 mu. thyrotropin per ml. of serum, an average of about 0.2 mu./ml. would appear to be very probable.

On 2 occasions comparison was made between the thyrotropin concentration found using whole serum and an extract of that serum. In one (No. 5) much greater activity was found in the extract than in the serum. An explanation is not obvious but it could have a bearing on Postel's finding (23) of a thyroid inhibiting activity in an extract (apparently alpha globulins) of serum. No depression of blood I^{131} was found in the present study after the injection of any of the serum components, however.

Serum No. 6 was assayed with almost identical concentrations of activity being found in the whole serum and in the gamma globulin, viz. 0.13 and 0.15 mu./ml. respectively. This was lower than the activity found in the serum from the other myxedematous patients. It is noteworthy that this patient had an intravenous injection of an iodine containing organic chemical, for renal phelography, 3 hours before blood was withdrawn for assay purposes. There was very high iodine content of both the serum and the gamma globulin, and this might well have had an inhibiting effect on any thyrotropin present. Such inhibition has been demonstrated by *in vitro* techniques (8). The action of iodine *in vivo* is far from being clearly understood but opposition to the action of thyrotropin has been postulated and given some experimental backing (24, 25). An alternative theory is that iodine acts by inhibiting the pituitary secretion of thyrotropin (26). This could equally well explain the finding of low thyrotropin content of the serum of this patient.

The finding of thyrotropic activity persistently and solely with gamma globulin is at variance with the statement of Cohn (27) that it was with beta globulins, and the findings of Querido and his colleagues (28) that it was variably and unpredictably with Cohn fractions II, III and IV-4. The assay technique of the latter workers was not very sensitive and it may be that in the few cases they have reported of detecting thryotropin in serum, the very high concentration of the hormone led to accociation with other serum proteins. Roberts (29) found thyrotropin mainly with albumin but stressed he was using rats with abnormal blood concentrations as the result of thyrotropin-secreting tumors. It is interesting that thyrotropin of bovine pituitary origin, when added to serum, was found to travel on electrophoresis with gamma globulin (23).

The validity of a standard of bovine pituitary origin for the assay of the hormone in human blood was tested statistically on one occasion with

Cohn fraction II. The deviation from parallelism was not statistically significant. Attention has recently (30) been drawn to the difficulty of assaying chorionic gonadotropin and adrenocorticotropic hormone in blood extracts against standards from sources other than blood. With thyrotropin, the slope of the response curve in the assay of extracts of human urine specimens was reported (31) to be "consistently lower than the slope of the standard TSH curve." Otherwise the question of the identity of action of the present day standard of thyrotropin and the hormone in human blood does not appear to have been raised. The reasonably parallel responses obtained with standard thyrotropin and Cohn fraction II in the present study would favor such an identity, but a final answer will require the preparation of an extract of purity comparable with that of the standard.

Acknowledgments

I am grateful to Dr. E. B. Astwood for continual interest and advice in this work which was supported in part by funds from the American Cyanamid Co., and the U. S. Public Health Services (Grant No. A-612). Throughout all the experiments Miss S. Mothon gave invaluable assistance.

REFERENCES

1. Rénon, L. and A. Delille: *Compt. rend. Soc. de biol.* **65:** 499. 1908.
2. Junkmann, K. and W. Schoeller: *Klin. Wchnschr.* **11:** 1176. 1932.
3. Fellinger, K.: *Wien. Arch. inn. Med.* **29:** 375. 1936.
4. Bodart, F. and K. Fellinger: *Wien. klin. Wchnschr.* **49:** 1286. 1936.
5. Collard, H. B., F. H. Mills, F. F. Rundle and E. P. Sharpey-Schafer: *Clin. Sc.* **4:** 323. 1940.
6. De Robertis, E.: *J. Clin. Endocrinol.* **8:** 956. 1948.
7. Del Conte, E. and J. F. E. Vasena: *Ann. endocrinol.* **13:** 910. 1952.
8. Albert, A.: *Ann. New York Acad. Sc.* **50:** 466. 1949.
9. Purves, H. D. and W. E. Griesbach: *Brit. J. Exper. Path.* **30:** 23. 1949.
10. Adams, D. D. and H. D. Purves: *Endocrinology* **57:** 17. 1955.
11. McKenzie, J. M.: *Fed. Proc.* **16:** 87. 1957.
12. McKenzie, J. M.: *Proc. Soc. Exper. Biol. & Med.* **95:** 736. 1957.
13. Kunkel, H. G. and R. J. Slater: *Proc. Soc. Exper. Biol. & Med.* **31:** 677. 1952.
14. Gaddum, J. H.: *J. Pharm. & Pharmacol.* **6:** 345. 1953.
15. Hawk, P. B., B. L. Oser and W. H. Summerson: Practical Physiological Chemistry, 12th Ed. p. 417. J. & A. Churchill Ltd., London. 1949.
16. D'Angelo, S. A., K. E. Paschkis, A. S. Gordon and A. Cantarow: *J. Clin. Endocrinol.* **11:** 1237. 1951.
17. Gilliland, I. C. and J. I. Strudwick: *Brit. M. J.* **1:** 378. 1956.
18. Adams, D. D. and H. D. Purves: *Proc. Univ. Otago Med. Sch.* **34:** 11. 1956.
19. D'Angelo, S. A.: Personal Communication. 1957.
20. Gilliland, I. C. and J. I. Strudwick: *Clin. Sc.* **12:** 265. 1953.
21. Di George, A. M., S. A. D'Angelo and K. E. Paschkis: *J. Clin. Endocrinol.* **17:** 842. 1957.
22. Greer, M. A. and H. F. Shull: *J. Clin. Endocrinol.* **17:** 1030. 1957.
23. Postel, S.: *Endocrinology* **58:** 557. 1956.
24. Rawson, R. W., F. D. Moore, W. Peacock, J. H. Means, O. Cope and C. B. Riddell: *J. Clin. Invest.* **24:** 869. 1945.

25. Solomon, D. H.: *Metabolism V*: 667. 1956.
26. Loeser, A.: *Klin. Wchnschr.* **13**: 533. 1934.
27. Cohn, E. J.: *Am. Scient.* **33**: 61. 1945.
28. Querido, A. and L. D. F. Lameijer: *Proc. Roy. Soc. Med.* **49**: 209. 1956.
29. Roberts, S.: Ciba Foundation Colloquia on Endocrinology. Volume XI. 78. 1957.
30. Loraine, J. A.: Ciba Foundation Colloquia on Endocrinology. Volume XI. 19. 1957.
31. Greenspan, F. S., J. P. Kriss, L. E. Moses and W. Lew: *Endocrinology* **58**: 767. 1956.

NOTES AND COMMENTS

THE *IN VIVO* EFFECT OF PURITY AND MODE OF ADMINISTRATION OF GLUCAGON ON GLYCOGENESIS

ABSTRACT

Fasted, white rats were given simultaneous, intraperitoneal injections of glucose and either amorphous or crystalline glucagon. The animals were sacrificed three hours after the injections and the amount of glycogen deposited in the liver was measured.

When the amorphous, or 50% pure glucagon was administered, there was less glycogen found for the same time period when compared with the administration of the crystalline glucagon. The effect of the crystalline glucagon could be potentiated by injecting it subcutaneously.

Though the presence of glucagon in the pancreas has been surmised since 1923, only recently has a very pure preparation of this material been available (1). Prior to 1953, workers used either crude extracts of the pancreas or partially purified substances when studying glucagon effects. During studies initiated to investigate the *in vivo* influence of this particular pancreatic factor on glycogenesis in liver and muscle (2), it was noted that two lots of glucagon[1] produced different results. Contrary to what was expected, crystalline glucagon was less effective in depleting liver glycogen than was an amorphous product which was only 50% pure.

In an effort to elucidate the reason for this variation, it was also found that intraperitoneal and subcutaneous injections of glucagon led to significantly different results.

METHODS

The method of studying glycogenesis as reported previously (3), was followed with slight modifications. Female white rats of Wistar strain, weighing between 190 and 210 gm. were fasted for 24 hours. At the end of that time, they received an intraperitoneal injection of glucose in 10% solution at a dose level of 2 gm. per kg. of body weight. Simultaneously, glucagon, dissolved in bicarbonate buffer, pH 8.6, was injected either intraperitoneally or subcutaneously over the abdomen in concentration such that the volume injected was 1 cc. per 100 gm. of body weight. Control animals received buffer and glucose or saline. At the end of a predetermined time, the animals were anesthetized with an overdose of Sodium Nembutal. Residual fluid in the peritoneal cavity was aspirated and a sample of blood was drawn from the heart. The liver was then quickly excised for multiple extraction with 5% trichloroacetic acid according to the method of Carroll, *et al.* (4) for glycogen. The glycogen was precipitated from the acid with 5 volumes of absolute ethanol and measured with anthrone reagent.

RESULTS

Figure 1 presents the comparison of the effects of amorphous and crystalline glucagon on glycogenesis in the liver 3 hours after the intraperitoneal injection of glucose and glucagon. The figures above the columns refer to the fact that the amorphous preparation was given in the dosages of 1 and 5 mg. per kg. of body weight; and the crystalline glucagon was given at dose levels of 0.5 and 2.5 mg. per kg. of body weight, which on the basis of 100% purity would correspond to the dose levels of the 50% pure material, and 5 mg. per kg., which was used for comparison with the higher amorphous dose on the possibility that the amorphous was more than 50% pure.

At both dose levels of the less pure sample, there was a significant decrease from the

Received March 29, 1958.

[1] The gift of the glucagon from Dr. W. R. Kirtley, Lilly Research Laboratories is gratefully acknowledged. Amorphous glucagon was Lot No. 258-234B-40, and crystalline glucagon was Lot No. 258-234B-54-2.

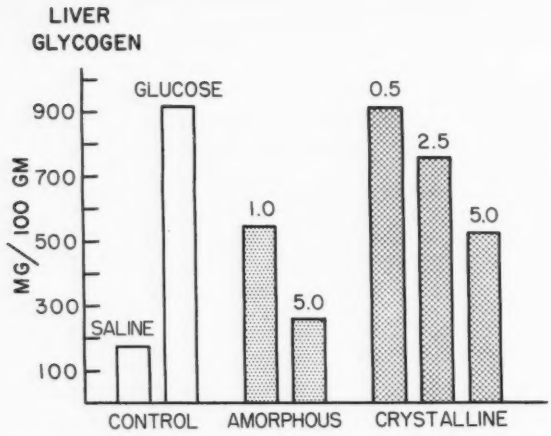


FIG. 1. Comparison of the effect on glycogenesis of amorphous and crystalline glucagon, 3 hours after intraperitoneal injection.

glycogen found when glucose alone was given. The glycogen levels of the animals treated with the crystalline glucagon were about two times as high as those receiving an equivalent dose of the amorphous product. Even the intraperitoneal injection of the crystalline glucagon at a level of 5 mg. per kg. did not cause as great a depression of liver glycogen as did the 5 mg. of the impure material. The difference between those two was significant ($P < .05$).

Since both lots of glucagon contained less than 0.05 U. of insulin per mg., another explanation was sought. It was further seen from the analyses of the peritoneal fluid that the absorption of glucose was similar under both conditions. Thus, it was felt that the difference could be explained on the basis of either a slower absorption from the peritoneal cavity or by a slower destruction by the tissues of the impure sample of glucagon; either or both effects might be due to the extraneous material present as impurity.

TABLE 1. COMPARISON OF INTRAPERITONEAL AND SUBCUTANEOUS ROUTE OF ADMINISTRATION OF CRYSTALLINE GLUCAGON

	Glucagon administered, mg./kg. body wt.	Liver glycogen, mg./100 gm. liver	
		Intraperitoneal	Subcutaneous
1 hour	none	440.4 ± 61.4 S.E. (9)	401.1 ± 83.5 S.E. (6)
	0.5	51.7 ± 26.3 (10)	7.2 ± 1.2 (8)
	5.0	7.7 ± 3.5 (5)	
3 hours	none	914.5 ± 106.9 (6)	953.3 ± 58.3 (7)
	0.5	910.0 ± 133.4 (8)	153.1 ± 28.1 (10)
	5.0	522.7 ± 58.0 (8)	4.3 ± 2.8 (7)

Figures in parentheses refer to number of animals.

However, from the work of Kenny (5) using partially pure preparations, and that of Narahara (6) using iodinated crystalline glucagon, in *in vitro* systems, it would appear that both are inactivated or degraded at relatively similar rates.

Table 1 shows the results obtained when the absorption of the crystalline glucagon is slowed by means of injecting subcutaneously. At 1 hour, there is not much differentiation, both modes of administration caused similar and significant depressions of liver glycogen. At 3 hours, the difference was obvious, with the subcutaneous route of injection potentiating the glycogen depressing effect considerably.

The possibility thus seems to exist that the presence of the impurity has a tendency to slow the absorption of the glucagon from the peritoneal cavity and potentiate its effects over a longer period of time. This might be due to the attachment of the active glucagon moiety to that of the impurity to form a much larger molecule which is only slowly absorbed.

ROBERT W. LONGLEY

Dorn Laboratory for Medical Research
Bradford Hospital
Bradford, Pennsylvania

REFERENCES

1. STAUB, A., L. SINN AND O. K. BEHRENS: *Science* **117:** 628. 1953.
2. LONGLEY, R. W. AND J. H. ROE: *Fed. Proc.* **16:** 214. 1957.
3. LONGLEY, R. W., A. L. SHIRKEY, O. H. ALVIG AND J. H. ROE: *Proc. Soc. Exptl. Biol. & Med.* **90:** 510. 1955.
4. CARROLL, N. V., R. W. LONGLEY AND J. H. ROE: *J. Biol. Chem.* **220:** 583. 1956.
5. KENNY, A. J.: *Am. J. Physiol.* **186:** 419. 1956.
6. NARAHARA, H. T. AND R. H. WILLIAMS: *Endocrinology* **60:** 285. 1957.

PRESENCE OF ABERRANT THYROID TISSUE IN THE GUINEA PIG[1]

ABSTRACT

Small highly active aberrant thyroid tissues were detected at various sites in thyroidectomized but not in normal guinea pigs of the Hartley and Zeimet strains by their ability to very actively accumulate I^{131}. Histological study showed follicles lined with flat cuboidal cells and the lumens filled with colloid.

Aberrant thyroids have been frequently detected as pathological growths at many different sites in man (1, 2). Studies utilizing serial histological examination of the thyroid area have indicated the presence of aberrant thyroids also in the guinea pig (3, 4, 5, 6), rabbit (3), dog (7, 8) baboon (9) and rat (10, 11). It seemed that a more detailed study of the distribution of aberrant thyroids could be made by the use of the sensitive I^{131} uptake technic.

PROCEDURE

Guinea pigs from three different sources were maintained in individual cages or in groups of 6 in an air-conditioned room. They were fed either Rockland guinea pig diet or the Jim Dandy Rabbit Pellets. A supplement of 25 mg. Vitamin C was fed weekly to each animal.

The thyroids were removed under ether anesthesia; special care was taken to clean the isthmus area. After thyroidectomy, a 1% solution of calcium gluconate was substituted for the drinking water for 10–14 days in order to prevent parathyroid deficiency. The animals showed (a) no change, (b) a cessation or (c) a decrease in growth rate for the first few days after thyroidectomy then resumed the normal growth rate.

The I^{131} solution (Abbott) was diluted so that the dose was contained in 1 ml. it was

Received April 15, 1958.

[1] This investigation was supported by research grants, A-914 and A-1726, from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

TABLE 1. THE PRESENCE OF ABERRANT THYROIDS IN THYROIDECTOMIZED GUINEA PIGS[a]

Expt.	Strain	Guinea pigs			I^{131}	Autopsy	Number of animals with aberrant thyroids at					
		No.	Age, days	B. wgt., gm.	μc.	Days after I^{131}	Tongue	Hyoid	Larynx	Submax. gland	Peri-tracheal	Thymus
					Normal							
1	Local	4	?	800	100	5						
2	Zeimet	2	145	950 700[b]	300	4						
3	Zeimet	6	140	900	300	3						
5	Hartley	7	99	640	300	3		1				
					Thyroidectomized							
2	Zeimet[c]	5	145	960	300	4	1[g]		3[i]	1	4[e]	1
4	Zeimet[c]	5	142	930	490	7					4[e]	3
5	Hartley[d]	7	99	600	300	3	3[g]	4[h]			2[f]	3

[a] The administration of the I^{131} and the determination of the radioactivity were supervised by Mr. W. W. Evans, University of Oklahoma, for experiments 1 and 2 and by Mr. Marshall Little, using the facilities of the radioisotope service of the Veterans Administration Hospital, Birmingham for Experiments 3 and 4.
[b] Castrated.
[c] Autopsy 47 days after thyroidectomy.
[d] Autopsy 37 days after thyroidectomy.
[e] In area of thyroid isthmus.
[f] Adjacent to trachea.
[g] At root of tongue. No discreet nodules. (thyroglossal duct?).
[h] At insertion of sternohyoid muscle.
[i] Probably at arytenoid cartilage (cf. 3). No discreet nodules.

administered intraperitoneally except in experiment 1 when it was administered subcutaneously (Table 1). At autopsy a sample of blood and all of the organs were separately exposed to a sodium iodide scintillation counter attached to a ratemeter or scaler. The aberrant thyroids were not grossly discernible but were localized by dissection until only the radioactive tissue remained. The tissues were fixed in 10% formaldehyde for histological study. In one experiment (Table 2) the radioactivity of the thyroids and aberrant thyroids was determined in a well-counter (Model BS-3, Nuclear-Chicago) which had a 41% efficiency for a 1 ml. solution of I^{131}.

RESULTS

Normal Guinea Pigs: In only one of the 19 guinea pigs was there any indication of aberrant thyroid tissue (Table 1). A small amount of radioactivity was detected at the insertion of the sternohyoid muscle but a discrete nodule could not be localized (Table 2).

The thyroid glands showed the expected uptake of I^{131} (Table 2).

Thyroidectomized Guinea Pigs: Each of the thyroidectomized guinea pigs possessed at least one aberrant thyroid (Table 1). The frequency and distribution (Fig. 1) of the aberrant thyroids differed slightly between the two strains of guinea pigs. The occurrence in and adjacent to the thymus was common for both strains. The Hartley strain showed

TABLE 2. WEIGHT AND I^{131} UPTAKE OF NORMAL AND ABERRANT THYROIDS OF THE MALE GUINEA PIG (HARTLEY)[a]

Guinea pig No.	Body wgt. change, gm.	Thyroid		Thymus		Peritracheal nodes		Sternohyoid		Tongue		Sum of total I^{131}
		Mg.	Total I^{131}	Mg.	Total I^{131}	Mg.	Total I^{131}	Mg.	Total I^{131}	Mg.	Total I^{131}	
					Normal Guinea Pigs							
(Ave.)	+134 (98–159)	90 (62–145)	7.8 (6.4–8.9)					—[b]	0.04			0.04
					Thyroidectomized Guinea Pigs							
656	+ 71			4	0.87							0.87
657	+ 56									(3)[b]	0.06	0.06
659	+ 80							—[b]	2.1	(?)[b]	(++++)[b]	2.1+
662	+ 76			3	1.07			11	3.3			4.4
666	+131					25[c]	2.1					2.1
669	+ 95			18	7.8							7.8
670	+180					10	2.5	25	6.3	(1)[b]	1.3	10.1

[a] These data are from the animals of Expt. 5, Table 1. The I^{131} values are expressed as per cent of the injected dose for the whole tissue.
[b] Discrete nodules could not be isolated.
[c] Two nodules, 10 and 15 mg.

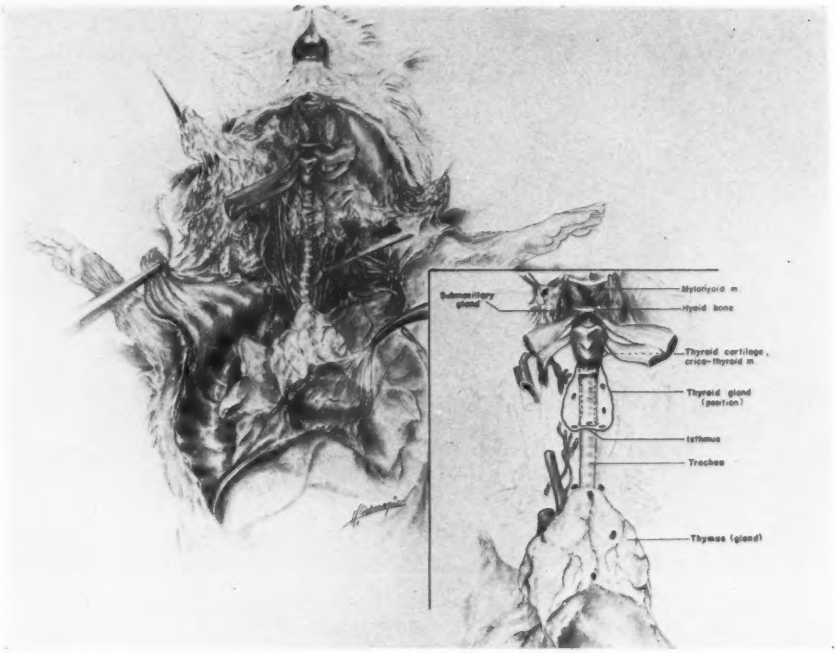


FIG. 1. Sites of aberrant thyroid tissue (indicated by black dots) in the thyroidectomized male guinea pig. (See Table 1). Discrete nodules could not be isolated in the region of the base of the tongue (thyroglossal duct?) or the thyroid cartilage in spite of the high uptakes of I^{131}.

aberrant thyroids at the insertion of the sternohyoid muscle while the Zeimet strain showed a comparable number at the larynx. The thyroid nodules near the trachea of the Zeimet strain occurred in the thyroid isthmus area and could possibly be due to incomplete removal of the isthmus which usually is a very thin and almost indiscernible strand. In other studies, small nodules have been occasionally noted adjacent to the isthmus.

The aberrant thyroids were readily dissected as discrete nodules except for those in the base of the tongue region and in the vicinity of the larynx. The weight of the nodules varied considerably but all were much smaller than the normal thyroids. The total I^{131} uptake for two of the animals, however, was equivalent to that of the normals and varied between 0.8 and 56 per cent of that of the normals for the remaining five guinea pigs. The increase in body weight of these animals also was below that of the normals. Histological study showed the nodules to be thyroid tissue (Fig. 2).

DISCUSSION

The use of the I^{131} technic reveals that aberrant thyroids may exist in more sites than previously recognized. The presence of aberrant thyroids in the peritracheal area (3–6) and near the larynx (arytenoid cartilage (3)) has been confirmed and the following new sites established: (a) in and adjacent to the thymus, (b) at the insertion of the sternohyoid muscle, (c) adjacent to the submaxillary gland and (d) at the base of the tongue (possibly the thyroglossal duct). The distribution of aberrant thyroids in the guinea pig now appears to be similar to that observed in man (1, 2).

The inability to detect aberrant thyroids in the normal guinea pig by the I^{131} technic is in agreement with the histological studies (3). The normal female rat of the Sprague-

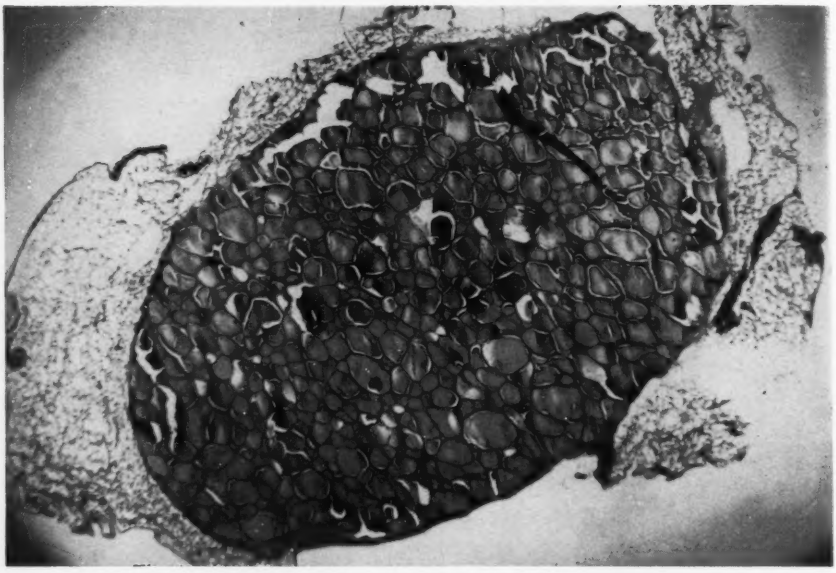


FIG. 2. Thyroid nodule found adjacent to submaxillary gland (cf. Fig. 1). Stained with hematoxylin and eosin. Magnification 9×.

Dawley strain, however, has been reported (11) to possess a high incidence of aberrant thyroid in the thymus.

The development of the highly active nodules in the thyroidectomized guinea pigs may explain the failure to note a decrease in the growth rate of these animals (12, 13). Goitrogens, however, also do not change the growth rate of guinea pigs (14, 15).

CHARLES D. KOCHAKIAN AND DON COCKRELL

University of Alabama Medical Center
Birmingham, Alabama

REFERENCES

1. MEANS, J. H. Thyroid and Its Diseases, Second Edition, p. 488, J. B. Lippincott Company, 1948.
2. GOLDSMITH, E. D.: *Ann. N. Y. Acad. Sci.* **50:** 283. 1949.
3. LEPORATI, I.: *Boll. d. Soc. Med. Chir. Pavia.* **51:** 1089. 1937.
4. SPODAFINA, L.: *Boll. Sur. Mel.* **8:** 619. 1929.
5. LOCATELLI, P.: *Boll. Soc. Med. Chir.* Pavia, 1935.
6. EITEL, H. and A. LOESER. *Arch. fur. exp. Pathologi u. Pharmacologie* **167:** 381. 1932.
7. GODWIN, M. C.: *Anat. Rec.* **66:** 233. 1936.
8. GRAHAM, A. WEST: *J. Surg.* **54:** 428. 1946.
9. VAN DYKE, J. H.: *Arch. Path.* **54:** 248. 1952.
10. VAN DYKE, J. H. *Arch. Path.* **55:** 412. 1953.
11. ASHLING, C. W., P. W. DURBIN, M. W. PARROTT, M. E. JOHNSTON AND J. C. HAMILTON: *Proc. Soc. Exptl. Biol. Med.* **94:** 200. 1957.
12. SIEBERT, W. J. AND R. S. SMITH: *Am. J. Physiol.* **95:** 396. 1930.
13. KOCHAKIAN, C. D. AND C. TILLOTSON: *Am. J. Physiol.* **189:** 425. 1957.
14. BROWN, M. N. AND W. C. YOUNG: *Endocrinology* **50:** 600. 1952.
15. HUMPHREYS, E. M.: *J. Endocrinol.* **8:** 220. 1952.

THE IMPLANTATION OF NEO-NATAL PANCREAS INTO THE CHEEK POUCH OF THE ALLOXAN DIABETIC HAMSTER

ABSTRACT

Neo-natal hamster pancreas, in which beta-cells were just beginning to differentiate, was implanted into the cheek pouches of both normal and alloxan-diabetic hamsters. It was found that: among the diabetics, 90% of the implants "took" while 75% "took" in the non-diabetics; the implants grew 30% larger and persisted 50% longer in diabetics than in normals; there was evidence for considerably more beta differentiation in the diabetic than in normals. Among the diabetics, where the graft persisted, blood sugars returned to the normal range and the pancreas of the host showed normal histology within two weeks of time the implants were made. Both of these latter events are considered rare for this particular post-alloxan period.

Although numerous attempts to transplant endocrine tissue into both normal and deficient hosts have been reported, only one (1) appeared to be of a definitely permanent nature. Even though it was shown that grafts are more likely to grow larger and survive longer if hosts are severely deficient (2 & 3), the consistent record of ultimate failure is a stimulus for a re-evaluation of the entire problem.

In 1952 (4) the hamster cheek pouch, as a site readily accessible for frequent examination of the growth of transplanted tissue, was described. Subsequently, it has been shown that even heterologous tissue will survive and grow long enough in this location to give valuable information. More recently, it was established that hamsters do not react as violently to foreign protein and that skin grafts persist longer in these animals than in other mammals (5). Supported by this evidence, it seemed logical to create an endocrine-deficient state and observe the growth of an implant, using the latter, for the first time, as a therapeutic measure in the alleviation of hyposecretory symptoms. Since it has been demonstrated that hamsters can be rendered diabetic by alloxan (6) and that they retain the potential to regenerate new islets (7 & 8) almost indefinitely, it was decided to use the alloxan-diabetic hamster as the host for the implantation of pancreatic tissue.

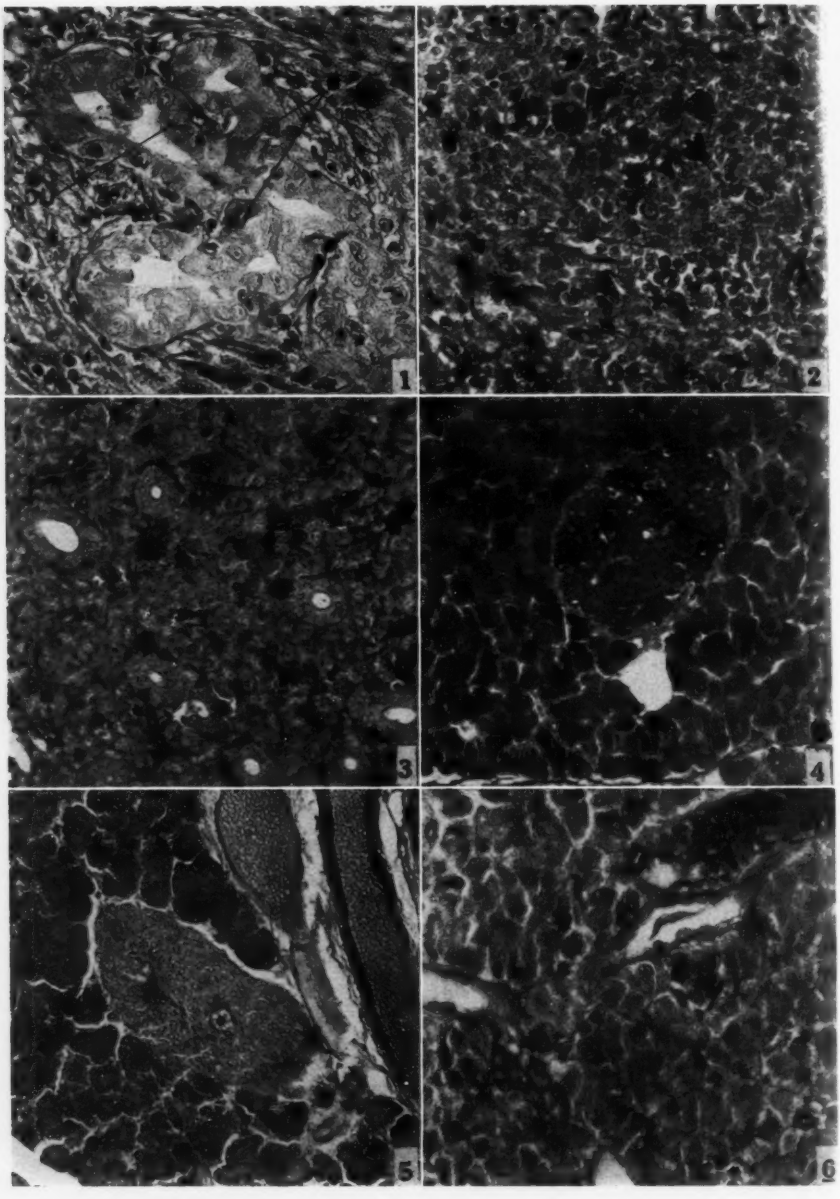
METHODS

As a preliminary step, a histological study was made of a series of fetal and neo-natal pancreas, beginning with the thirteenth fetal and extending to the twenty-first post-natal day. Later a group of hamsters, 8 to 9 weeks old, were injected intravenously (9) with a diabetogenic dose of alloxan (10). These were checked periodically for glucose by a modified anthrone technique (11). Since it had been demonstrated that animals still diabetic 4 weeks after alloxan will remain hyperglycemic for at least 4 weeks longer (8), only hamsters showing high blood glucose values at the end of a month were used as hosts. The latter were implanted with pancreas from new-born animals 4 to 6 hours after birth. The amount of growth was observed at intervals and blood sugars were run every third or fourth day. A few animals were sacrificed, their tissues fixed in Bouin's fluid and stained by our own modification of Gomori's aldehyde fuchsin (12). A small number of non-diabetic hamsters of comparable age were also implanted with pancreas from similar donors.

OBSERVATIONS

An examination of the developmental stages showed: that beta cell differentiation begins 4 to 6 hours after birth; that differentiation reaches its greatest activity on the fourth post-natal day when the islets are relatively larger, more numerous and more heavily granulated than at any other time; that islets have attained essentially adult characteristics by the fourteenth day, although new differentiation, particularly from the small ducts, is still very active at this time; that differentiation has tapered off to adult levels by the twenty-first day. When the implants in the diabetics were compared with the non-diabetic, it was found that: among the diabetics 90% "took" while in the normals 75% "took"; the growth in volume was 30% more and the survival time was 50% longer in the diabetics. An examination of sections indicates that beta differentia-

Received May 31, 1958.


1
2
3
4
5
6

tion occurs in implants of both diabetic and non-diabetic hosts but to a very limited extent in the latter (Fig. 1). A section through the graft of a diabetic animal whose pouch showed gross evidence of atrophic changes during the third week shows that a considerable amount of differentiation had taken place before regression began (Fig. 2). However, it is evident, from the examination of a sample taken from a diabetic animal whose implant appeared in good condition after 14 days, that differentiation can become very extensive (Fig. 3). Blood glucose analysis performed on the diabetic animals showed a return to normal values within two weeks after implantation. However, the most interesting finding was the fact that, in all of the diabetics in which the graft survived as long as two weeks, the pancreas of the host had returned to histological normal during that time (Fig. 4). For comparison, two typical sections from other hamsters, diabetic for comparable periods, but otherwise untreated, have been included (Figs. 5, 6).

The results of this pilot experiment are being used as a guide for future investigations.

E. L. House, C. Burton,
H. Cooper and E. Anderson

Department of Anatomy
New York Medical College

REFERENCES

1. Broster, L. R.: *Med. Illustrated* **7**: 389. 1953.
2. Dameron, J. T.: *Surg. Forum* p. 570. 1950.
3. Woodruff, M. F. A.: *Ann. Roy. Col. Surg.* **11**: 73. 1952.
4. Lemon, H. M., B. R. Lutz, R. Pope, L. Parsons, A. H. Handler and D. I. Patt: *Science* **115**: 461. 1952.
5. Adams, R. A., D. I. Patt and B. R. Lutz: *Trans. Bul.* **3**: 41. 1956.
6. Harris, P. N., R. C. Anderson and K. K. Chen: *J. Pharm. and Exp. Therap.* **87**: 382. 1946.
7. House, E. L. and J. P. Tassoni: *Endocrinology* **61**: 309. 1957.
8. House, E. L.: *Endocrinology* **62**: 189. 1958.
9. Tassoni, J. P.: *Anat. Rec.* **121**: 453. 1955.
10. Nace, P. F., E. L. House and J. P. Tassoni: *Endocrinology* **58**: 305. 1956.
11. Durham, W. F., W. L. Bloom, G. T. Lewis and E. E. Mandel: *Pub. Health Rep.* **65**: 670. 1950.
12. House, G. M. *Unpublished data.* 1955.

Fig. 1. Implant from non-diabetic whose pouch showed evidence of atrophy near the end of first week. Shows branching duct "D" containing an occasional beta-cell, "B." Aldehyde fuchsin. ×425.

Fig. 2. Implant from diabetic whose pouch showed atrophic changes near the end of the third week. Dark cells contain beta-granules. Aldehyde fuchsin. ×425.

Fig. 3. Implant from diabetic whose pouch was in excellent condition after 14 days. Irregular, dark granular patches represent masses of beta-granules. Aldehyde fuchsin. ×145.

Fig. 4. Pancreas from host, diabetic 4 to 5 weeks before grafting and sacrificed 2 weeks later. This is an essentially normal islet. Aldehyde fuchsin. ×145.

Fig. 5. Pancreas from an animal diabetic 6 to 7 weeks but not implanted. No beta-cells are evident in this or other islets. Aldehyde fuchsin. ×145.

Fig. 6. Pancreas from an animal diabetic 7 to 8 weeks but not implanted. This shows regeneration of two small nests of beta-cells from walls of small ducts. No normal medium or large islets in the entire pancreas. Aldehyde fuchsin. ×425.

CONVERSION OF Δ^5-ANDROSTENE-3β,16α,17β-TRIOL TO ESTRIOL BY HUMAN PLACENTA[1]

ABSTRACT

The *in vitro* conversion of Δ^5-androstene-3β,16α,17β-triol to estriol by human placental preparations has been described. Estriol was characterized by determining several physical constants of the isolated product and its methyl ether, as well as by a positive Bachman reaction. This unique conversion suggests a biological role for the neutral 16-hydroxylated steroids previously isolated from human urine and lends support to the concept of a biosynthetic pathway for estriol not involving known estrogen precursors.

During the course of studies on the *in vitro* biosynthesis of estrogens, a highly efficient human placental system was described which converted Δ^4-androstene-3,17-dione to estrone and testosterone to estradiol (1, 2). The production of estriol, however, was not detected with these substrates. Human placenta is almost certainly one source of estriol, but no direct evidence for its *in vitro* synthesis by this organ has as yet been provided. The present report describes the *in vitro* conversion by human placental preparations of a known human urinary metabolite, Δ^5-androstene-3β,16α,17β-triol (3, 4), to a product with the characteristics of estriol.

MATERIALS AND METHODS

Term human placentas were obtained on delivery and prepared according to methods previously described (1, 2). The 10,000×g supernatant fraction containing microsomes and the soluble enzymes was used exclusively. 50 cc. Flasks containing the placental preparations, cofactors, and the steroid substrate, dissolved in propylene glycol, were agitated for one hour in air at 37° C in a Dubnoff incubator.

The incubation mixtures were extracted with six volumes of $CHCl_3$ three times and the $CHCl_3$ taken to dryness. The $CHCl_3$ residue was freed of lipid by a pentane/90% methanol partition and the methanol extract subjected to a phenol separation (5). The phenolic fraction was used for assay, further purification and characterization of the product.

Fluorescence determinations were by the method of Engel *et al.* (5) or a modification of the procedure described by Sweat (6). Kober chromogens were determined according to Brown (7). Paper chromatography was carried out according to the method of Bush (8) with two modified solvent systems: ligroine:toluene (2:1)/70% methanol and toluene/75% methanol. Compounds were identified on paper with the ferric chloride-potassium ferricyanide reagent. The Bachman reaction was performed on paper as described by Axelrod (9), and countercurrent distribution carried out according to the method of Engel (5). Methylation and purification of the methyl ethers have been described by Brown (7). The methyl ethers were determined by fluorescence or the Kober reaction and detected on paper by a phosphomolybdic acid spray (5% in ethanol).

The Δ^5-androstene-3β,16α,17β-triol was obtained from Dr. H. Hirschmann through Dr. L. Engel and was demonstrated to be pure by paper chromatography. Infra red analysis of the sample was carried out by Dr. L. Engel. The number of experiments performed and hence the amount of estriol produced was limited by the availability of the substrate.

RESULTS

No estriol was produced in control experiments in which substrate was omitted or in which extraction was carried out at zero time. Incubation of the complete system with Δ^5-androstene-3β,16α,17β-triol for one hour produced the 12% conversion to estriol described in Table 1. Based on similar studies with dehydroepiandrosterone[2] it is believed that the reaction proceeds from the Δ^5-compound via a Δ^4-3-ketone to estriol. Presumptive evidence of such a pathway is available from the production of an ultraviolet absorbing compound in the incubation experiments, but this possible intermediate has not as yet been isolated.

Received May 20, 1958.

[1] This investigation was supported by a grant from the Josiah Macy, Jr. Foundation to Harvard Medical School.

[2] Unpublished data.

TABLE 1. CONVERSION OF Δ^5-ANDROSTENE-3β,16α,17β-TRIOL TO ESTRIOL

System	Number of preparations	Average μg. estriol produced
Minus substrate, incubated	8	0
Complete system, zero time	4	0
Complete system, incubated	4	31

Conditions: Placental 10,000×g. supernatant fraction equivalent to 11 grams wet weight of tissue, 250 μg. Δ^5-androstene-3β,16α,17β-triol and the following in μM. per cc.: phosphate buffer pH 7, 50, niacinamide 40, ATP 10 and DPN 10 in a total volume of 5 cc. incubated for one hour in air at 37° C.

The estriol produced was characterized in the following manner. Paper chromatography in the two modified Bush systems revealed Rf values identical with estriol standards

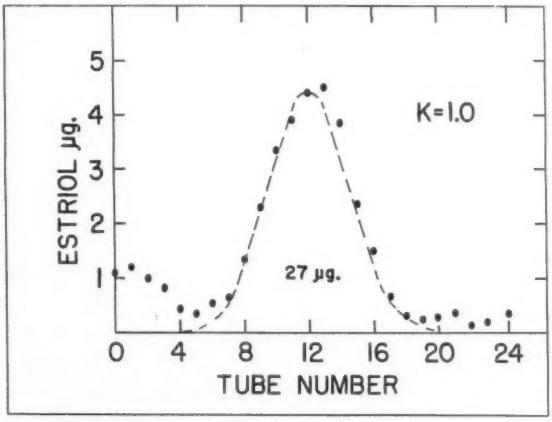


FIG. 1. 24-Transfer countercurrent distribution in 70% methanol/$CHCl_3$:CCl_4 (4:1) of the product obtained after incubating Δ^5-androstene-3β,16α,17β-triol with human placenta. *K* is the partition coefficient calculated from the experimentally determined fluorescence values (closed circles), and the broken line represents the theoretical distribution curve.

run at the same time. The Bachman reaction was positive.[3] A 24 transfer countercurrent distribution in 70% methanol/$CHCl_3$:CCl_4 (4:1) provided the curve shown in Figure 1. Agreement between experimental points and the theoretical distribution is acceptable, and the partition coefficient obtained was identical with that of pure estriol.[4] Peak tubes of the distribution were pooled and methylated and the derivative purified by solvent partition (7). The methylated derivative had a partition coefficient in 60% methanol/CCl_4 identical with pure Estriol methyl ether and migrated in the toluene/75% methanol Bush system with an Rf equivalent to a similarly run standard. There was insufficient material for infra red analysis or melting point determinations, but the physical characteristics demonstrated for the product and its methyl ether, as well as the positive

[3] The author is indebted to Dr. O. Smith for assistance with this procedure.

[4] Facilities for the countercurrent distribution were generously provided by Dr. L. L. Engel.

Bachman reaction, provide reasonably strong evidence for its identity as estriol short of these more definitive criteria. Diczfalusy has employed similar methods to establish the identity of small amounts of endogenous placental estrogens (10).

DISCUSSION

The formation of estriol from estrone and estradiol has been well documented by *in vivo* studies (11), and is considered the major source of estriol production. The present work suggests that estriol can also be produced from a non-estrogen precursor without involving the classic conversion from estrone and estradiol. Hisaw has postulated such a possibility on the basis of several prior lines of evidence (12). Since the starting material is one of a family of normally occurring urinary steroids (13), a potential metabolic role for the 16-hydroxylated neutral compounds may now be suggested. Further *in vivo* and *in vitro* studies will be necessary to determine the relative significance of this conversion of Δ^5-androstene-3β,16α,17β-triol to estriol in terms of the synthetic and catabolic pathways of the estrogens and the unique biological role estriol plays in comparative endocrinology and pregnancy.

The author wishes to express his appreciation to Mr. Leonard Bushnell for technical assistance.

KENNETH J. RYAN

Boston Lying-in Hospital and
Department of Obstetrics
Harvard Medical School
Boston, Massachusetts

REFERENCES

1. RYAN, K. J.: *Biochim. Biophys. Acta* **27**: 658. 1958.
2. RYAN, K. J.: *Fed. Proc.* **17**: 138. 1958.
3. HIRSCHMANN, H.: *J. Biol. Chem.* **150**: 363. 1943.
4. MARRIAN, G. F.: *Nature* **154**: 19. 1944.
5. ENGEL, L. L., W. R. SLAUNWHITE, JR., P. CARTER AND I. T. NATHANSON: *J. Biol. Chem.* **185**: 255. 1950.
6. SWEAT, M. L.: *Anal. Chem.* **26**: 773. 1954.
7. BROWN, J. B.: *Biochem. J.* **60**: 185. 1955.
8. BUSH, I. E.: *Biochem. J.* **50**: 370. 1952.
9. AXELROD, L. R.: *J. Biol. Chem.* **201**: 59. 1953.
10. DICZFALUSY, E. AND P. LINDKVIST: *Acta Endocr.* **22**: 203. 1956.
11. DORFMAN, R. I.: *Annual Rev. Biochem.* **26**: 523. 1957.
12. HISAW, F. L., J. T. VELARDO AND C. M. GOOLSBY: *J. Clin. Endocrinol. and Metab.* **14**: 1134. 1954.
13. LIEBERMAN, S., B. PRAETZ, P. HUMPHRIES AND K. DOBRINER: *J. Biol. Chem.* **204**: 491. 1953.

THE ENDOCRINE SOCIETY

1959 Annual Meeting

The Forty-First Annual Meeting of The Endocrine Society will be held in the Haddon Hall Hotel, Atlantic City, New Jersey, Thursday, Friday, and Saturday, June 4, 5, 6, 1959.

The Chairman of Local Arrangements is Dr. Matthew Molitch.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 5th at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Chalfonte-Haddon Hall Hotel will hold 300 bedrooms for members until May 1, 1959, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. Make your reservations now and avoid disappointment.

Final program, membership card and advance registration forms will be sent on May 1st 1959 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Frank Engel, Duke Hospital, Durham, North Carolina, not later than February 1, 1959. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. The Council requests that authors adhere strictly to the following rules when submitting abstracts, otherwise they cannot be considered:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.

2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

4. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

5. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1959 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was elected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1958.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1958.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

CORRECTION

In the article by S. M. McCann, A. Fruit and B. D. Fulford entitled "Studies on the Loci of Action of Cortical Hormones in Inhibiting the Release of Adrenocorticotrophin" which appeared in the July, 1958 issue of ENDOCRINOLOGY the two references to Fig. 5 in Footnote 3 on page 36 should each read Fig. 4. And, in the legend of Figure 3, page 35, the last sentence should read as follows: Dose: The open circles represent rats injected with 1 mg./day; the solid circles those injected with 2 mg./day; and the crosses those injected with 4 mg./day.